Workshop Summary

The Human Microbiome, Diet, and Health

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Food Forum

Food and Nutrition Board

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Willing is not enough; we must do."
—Goethe

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This report has been reviewed in draft form by individuals chosen for their diverse perspectives and technical expertise, in accordance with procedures approved by the National Research Council's Report Review Committee. The purpose of this independent review is to provide candid and critical comments that will assist the institution in making its published report as sound as possible and to ensure that the report meets institutional standards for objectivity, evidence, and responsiveness to the study charge. The review comments and draft manuscript remain confidential to protect the integrity of the process. We wish to thank the following individuals for their review of this report:

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Although the reviewers listed above have provided many constructive comments and suggestions, they did not see the final draft of the report before its release. The review of this report was overseen by **Melvin Worth.** Appointed by the Institute of Medicine, he was responsible for making certain that an independent examination of this report was carried out in accordance with institutional procedures and that all review comments were carefully considered. Responsibility for the final content of this report rests entirely with the authors and the institution.

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Overview

The Food Forum convened a public workshop on February 22-23, 2012, to explore current and emerging knowledge of the human microbiome, its role in human health, its interaction with the diet, and the translation of new research findings into tools and products that improve the nutritional quality of the food supply. This report summarizes the presentations and discussions that took place during the workshop.[1] Box O-1 provides definitions of the human microbiome and other key terms used throughout this report.

Several major overarching themes emerged over the course of the 2-day dialogue:

- The microbiome is integral to human physiology, health, and disease.
- The microbiome is arguably the most intimate connection that humans have with their external environment, mostly through diet.
- Given the emerging nature of research on the microbiome, some important methodology issues might still have to be resolved with respect to undersampling (i.e., some workshop participants expressed

[1] The workshop was organized by an independent planning committee whose role was limited to designing the workshop program and identifying goals, topics, and speakers. This workshop summary has been prepared by the rapporteurs as a factual summary of the presentations and discussions that took place at the workshop. Statements, recommendations, and opinions expressed are those of individual presenters and participants and are not necessarily endorsed or verified by the Food Forum or the National Academies; they should not be construed as reflecting any group consensus.

concern not just about underpowered studies, but also tissue undersampling) and a lack of causal and mechanistic studies.
- Dietary interventions intended to have an impact on host biology via their impact on the microbiome are being developed, and the market for these products is seeing tremendous success. However, the current regulatory framework poses challenges to industry interest and investment.

In her keynote address, Karen Nelson, president of the J. Craig Venter Institute (JCVI), touched on all of these themes. With respect to the in-

BOX O-1
Definition of Key Terms

Commensal: An organism participating in a symbiotic relationship in which one species derives some benefit while the other is unaffected

Enterotype: The concept that distinct communities of bacteria are defined by their bacterial composition (Arumugam et al., 2011)

Metabonomics: The quantitative measurement of the multiparametric (time-related) metabolic responses of complex systems to a pathophysiological stimulus or genetic modification (Nicholson et al., 1999); often used synonymously with **metabolomics** (Fiehn, 2002)

Metagenomics: The study of the gene content and encoded functional attributes of the gut microbiome in healthy humans (Gill et al., 2006)

Microbiome (human): The full complement of microbes (bacteria, viruses, fungi, and protozoa), their genes, and genomes in or on the human body

Prebiotic: A substance that (1) is resistant to gastric acidity, to enzymatic hydrolysis, and to gastrointestinal absorption (i.e., not hydrolytically digestible); (2) is fermented by cecal-colonic microflora; and (3) selectively stimulates growth and/or activity of those bacteria that contribute to colonic and host health (Gibson et al., 2004) *or* a nonviable food component that confers a health benefit on the host associated with modulation of the microbiota (Pineiro et al., 2008)

Probiotics: Living microorganisms that when administered in adequate amounts confer a health benefit on their host (FAO-WHO, 2002)

Resistome: The collective informational resources available to the microbiome for responding to antimicrobial pressure (Wright, 2007)

tegral role of the microbiome in human physiology, health, and disease, she described some of the findings that JCVI scientists have made in their studies on gut microbiome-disease associations (Fouts et al., 2012; Yan et al., 2011). For example, JCVI scientists are working in collaboration with researchers from New York University to examine how the microbiome changes over time in individuals with esophageal cancer. The researchers are detecting unique microbial signatures associated with different stages of esophageal cancer. She also described some of the work that JCVI researchers have been doing on fundamental microbiome functioning (e.g., how microbial gene expression varies depending on what other species are present) and JCVI efforts to access once-inaccessible genomic information that can be used to help develop novel nutritional (e.g., probiotic) tools. Nelson's talk prompted a lively discussion about methodology, mostly about the limitations of undersampling. JCVI researchers are credited with laying much of the conceptual and technological groundwork for contemporary research on the microbiome (e.g., Eckburg et al., 2005; Gill et al., 2006; Human Microbiome Jumpstart Reference Strains Consortium, 2010; Rusch et al., 2007; Venter et al., 2004; Wu et al., 2011a; Yooseph et al., 2007).

STUDYING THE MICROBIOME

While study of what is now known as the human microbiome can be traced as far back as Antonie van Leeuwenhoek (1632-1723), advances in genomics and other areas of microbiology are driving the field in a direction van Leeuwenhoek could not have imagined. Although scientists are increasingly shifting their attention toward studying not just what microbes are present in (and on) the human body, but also what those microbes are doing, the field still revolves around genomics. A major goal of the Human Microbiome Project (HMP) is to characterize the genomic makeup of all microbes inhabiting the human body. Lita Proctor, coordinator of the National Institutes of Health Common Fund HMP, explained how HMP researchers are building a publicly available reference database of microbiome genomes from "healthy," or "normal," individuals, with the intention of providing researchers with "healthy cohort" information for use in comparison studies. The HMP is also coordinating a series of "demonstration projects" aimed at identifying characteristic microbial communities associated with certain human diseases (e.g., an enrichment of *Fusobacteria* with colorectal cancer).

Based on what the HMP and other investigators have observed, Proctor elaborated on what she views as "universal" properties of the microbiome, that is, properties shared by all hosts. In her opinion, most universal properties identified thus far have to do with the dynamic nature of the microbiome over time, or the way the microbiome changes in composition over the

course of a human lifetime. For example, one key universal property is that unlike the human genome, the human microbiome is acquired anew each generation, with vaginally born babies acquiring different microbiomes than cesarean section (C-section) babies (Dominguez-Bello et al., 2010). Meanwhile, Proctor questioned whether certain other phenomena—namely, enterotypes, the notion of a "core" microbiome, and the idea that the presence of a pathogen indicates disease—are universal properties. None of these, in her opinion, are universal properties based on the evidence to date (e.g., Wu et al., 2011a).

Following Proctor's presentation, Jennifer Russo Wortman, director of microbial informatics at the Broad Institute, described methodologies that HMP Consortium investigators are using to analyze the massive amount of genomic data that are accumulating. Most researchers are using one of two types of data: (1) 16S ribosomal ribonucleic acid (rRNA) data to determine what microbes are present (i.e., by using operational taxonomic units, or OTUs, as proxies for species) and (2) whole-genome shotgun reads to get a sense of what these microbes might be doing (i.e., by comparing sequences to functional databases). The data reveal varying levels of microbial diversity, depending on taxonomic level, among body sites (e.g., vaginal samples have less genus-level microbial diversity than other body sites, but more species-level diversity). Among individual hosts, scientists are observing greater compositional diversity (based on 16S rRNA reads) than putative functional diversity (based on shotgun reads). One of the greatest challenges in moving forward will be interpreting the massive amount of sequencing data that are accumulating, especially with respect to function, by integrating them with transcriptomic, proteomic, and metabolomic data into a systems-level approach to studying the microbiome.

Jeremy Nicholson, head of the Department of Surgery and Cancer at the Imperial College London, argued that not only is an integrative, systems-level approach necessary for understanding human health and disease, but studying the microbiome is central to that approach (Mirnezami et al., 2012; Nicholson, 2006). Only by understanding how gut microbes are signaling and otherwise functioning, especially with respect to their impact on their human host, will scientists ever be able to tease apart human biocomplexity enough to realize the vision of personalized health care. Nicholson discussed some of the ways that gut microbes influence human host metabolism and generate differential metabolic phenotypes (Holmes et al., 2008). For example, mouse and rat studies have demonstrated what Nicholson described as a "massive effect of the microbiome on bile acid metabolism," with gut microbial activity impacting liver and colonic disease risk as a result (Martin et al., 2007; Swann et al., 2011).

THE MICROBIOME, HEALTH, AND DISEASE

While demonstrated associations between the human microbiome and health or disease were an overarching theme of the workshop, with most speakers at least touching on the topic, some speakers homed in on it. Josef Neu, professor of pediatrics in the Division of Neonatology at the University of Florida, provided an overview of recent microbiome-disease research in pediatric populations. First, he described evidence suggesting that a fetal microbiome exists; that is, babies are born with microbiomes acquired during the last trimester of pregnancy (DiGiulio et al., 2008; Goldenberg et al., 2000; Koenig et al., 2011). The existence of a fetal microbiome has clinical implications, with greater microbial diversity being associated with prematurity (DiGiulio et al., 2008; Mshvildadze et al., 2010). Then he summarized recent evidence of associations between microbiome composition and two diseases prevalent among babies in neonatal intensive care units (ICUs): necrotizing enterocolitis and late-onset sepsis (Alexander et al., 2011; Mai et al., 2011). Neu also explored in more detail a topic that Lita Proctor had mentioned, that is, microbiome differences between babies born vaginally and babies born via C-section (Dominguez-Bello et al., 2010). The differences are important not only because of the increasing prevalence of C-section deliveries in many countries, but also because of the wide range of immune-related diseases associated with C-section delivery (Neu and Rushing, 2011). Finally, he remarked on other recent evidence indicating associations between microbial ecology in children and the onset of type 1 diabetes (Brown et al., 2011; Vaarala et al., 2008). Together, these various avenues of research suggest that the early microbiome, from fetal development through childhood, can influence both short- and long-term health.

Researchers have made significant headway in understanding how the oral microbiome contributes to health and disease. Richard Darveau, professor and chair in the Department of Periodontics at the University of Washington Dental School, described evidence indicating that unlike many other human pathogens, the periopathogen *Porphyromonas gingivalis* triggers disease not by inducing inflammation but by intervening with host immunity in a more subversive manner. In fact, inflammation is a normal part of a healthy oral environment, with neutrophil movement being a sign of healthy "immune surveillance" and cytokine production contributing to healthy tissue development and function (Roberts and Darveau, 2002). Eventually, over time, even a healthy mouth experiences bone loss. However, *P. gingivalis* accelerates the process. The bacteria interferes with innate immunity in a way that prevents the host from detecting and clearing not just *P. gingivalis*, but other oral microbes as well (Burns et al., 2010; Coats et al., 2005, 2007; Hajishengallis et al., 2008a,b, 2011; Liang et al.,

2011; Wang et al., 2010). Darveau said, "It actually takes something that is already functioning and modulates that."

Vincent Young, associate professor at the University of Michigan Medical School, expanded on the theme that disease reflects an imbalance in the microbiome. Using *Clostridium difficile* as an example, he discussed how medical thinking around infectious disease is shifting. When he was a medical student, the paradigm revolved around finding the lone "bad bug" and the "drug for bug." Young teaches his students to consider instead bad versus good *communities* of microbes. He described a series of experiments that he and colleagues have conducted to better understand what factors influence whether an indigenous gut microbiota resists or succumbs to *C. difficile* colonization and disease (Chang et al., 2008). Evidence suggests that *C. difficile* illness is a function of how resilient the indigenous microbiota is following an antibiotic assault, with some communities able to restore balance following withdrawal of the antibiotic and others not. Recurrence is also a common problem with *C. difficile*, with 25 percent of patients becoming sick again after ending antibiotic treatment due to continued imbalance of the gut microbiota. Restoring balance in the indigenous microbiota—for example, by adding a "good bug" or combination of "good bugs"—could be the basis for a novel therapeutic approach to managing *C. difficile* disease.

HOW THE MICROBIOME INFLUENCES HOST DIET METABOLISM

Although research on the microbiome is considered an emerging science, scientists already have made tremendous progress in understanding the microbial makeup of the microbiome and associating microbiome diversity with human disease. Moreover, they are beginning to make headway in understanding *how* the microbiome impacts human health and disease. It is likely that much of this impact is mediated through diet. Growing evidence suggests that gut microbes influence what the human host is able to extract from its diet, including energetically.

Peter Turnbaugh, Bauer fellow in the FAS Center for Systems Biology at Harvard University, summarized some of what is known about how the gut microbiome influences host energetics based on a series of mouse model studies demonstrating that gut microbes influence obesity (Backhed et al., 2004; Ley et al., 2005; Turnbaugh et al., 2006, 2008). For example, when the gut microbiota of obese mice is transplanted into germ-free mice, the mice gain more body fat compared to initially germ-free mice that receive microbiota transplants from lean mice; furthermore, the obese microbiome has been shown to extract more energy from the same amount of kilocalories compared to the lean microbiome (Turnbaugh et al., 2006, 2008). Other mouse data from Turnbaugh's lab suggest that the microbiome impacts host

metabolism in other ways as well. For example, he described work done in collaboration with Lee Kaplan's group at Massachusetts General Hospital utilizing a mouse model for gastric bypass surgery. These results highlight dramatic changes in the gut microbiota immediately following surgery. Researchers are now investigating which metabolic outcomes of surgery may be influenced by the gut microbiota.

Indeed, a growing body of evidence suggests that the microbiome impacts a wide range of host metabolic pathways. Using degradation of plant chemicals as an example, Johanna Lampe, associate division director in the Public Health Sciences Division at the Fred Hutchinson Cancer Research Center, explored the many roles that microbes play in host metabolism and how those microbial contributions influence disease prevention and disease risk (Qin et al., 2010; Scalbert et al., 2011). She highlighted the glucosinolates (the chemical precursors to a compound in cruciferous vegetables that protects against cancer) (Li et al., 2011; Shapiro et al., 2001), soy isoflavones (which have been associated with a variety of health outcomes in perimenopausal women) (Akaza et al., 2002; Atkinson et al., 2003; Frankenfeld et al., 2004; Fuhrman et al., 2008), and plant lignins (Kuijsten et al., 2005).

HOW DIET IMPACTS THE MICROBIOME

As the workshop progressed, speakers explored in greater depth the impact of diet on the microbiome; how dietary influences on the microbiome contribute to human health and disease; and ways to modulate the microbiome to build and maintain health through the use of prebiotics and probiotics in food products.

Diet-related diseases have become more prominent in today's society. For Bruce German, professor in the Department of Food Science and Technology at the University of California, Davis, that raises the question: Is it possible to prevent disease through diet? German's quest to understand the preventive potential of diet led him to "the one thing" that evolved to promote a reduction in risk: human breast milk. He described work by Carlito Lebrilla, David Mills, and others on the association between human milk oligosaccharides (HMOs) and *Bifidobacterium infantis*, a dominant member of the breast-fed-infant microbiome. HMOs are the third most predominant component of human breast milk (Wu et al., 2010, 2011b). Yet, they are undigestible by the infant. As it turns out, their role is to serve as a food source not for the infant, but rather for *B. infantis* (LoCascio et al., 2007; Marcobal et al., 2010; Sela et al., 2011; Ward et al., 2006, 2007). "The mother's milk is providing a growth medium for the bacteria," German said. Knowledge of the HMO-*B. infantis* association is also being used to explore new ways to improve the health of premature infants.

Sharon Donovan, professor and Melissa M. Noel Endowed Chair in Nutrition and Health at the University of Illinois, is hopeful that her research on the impact of a breast milk diet on the infant microbiota will help to develop new ways to improve the health of formula-fed infants. She wondered whether there might be substances that could be added to infant formula to provide formula-fed infants with the same health benefits afforded by breast-feeding. Using a noninvasive stool sampling methodology, she and colleagues have detected several significant differences in gene expression between breast-fed and formula-fed infants (Chapkin et al., 2010; Davidson et al., 1995). Moreover, they have correlated some of that variation with variation in host gene expression, providing clues about how diet-modulated microbial signaling affects host biology (Schwartz et al., 2012).

Although food may be the primary modulator of the microbiome, it is not the only modulator. Ellen Silbergeld, professor in epidemiology, environmental health sciences, and health policy and management at Johns Hopkins University, explained that the way most food animals are raised is another major driver of the microbiome. Specifically, extensive antibiotic use in the modern livestock farm exerts a selective pressure for antibiotic resistance that spreads beyond the farm to the ecosystem at large and eventually to the human microbiome. Silbergeld introduced the notion of a "resistome," which she defined as the collective informational resources available to the microbiome for responding to antimicrobial pressure (Wright, 2007). An important feature of the resistome is horizontal gene transfer. Because of the rapid and efficient transfer of resistance genes from one bacterium to another, even nonpathogenic (so-called commensal) bacteria can carry and express resistance genes. Thus, the microbiome is a major part of the resistome; in addition, naked DNA in ecological niches is available for internalization by competent bacteria. Silbergeld elaborated on the way the resistome expands across space—from food animals to the soil environment to the human gastrointestinal (GI) tract—and the implications for human health of antibiotic resistance in bacteria carried by food animals and often transferred to food during processing (Danzeisen et al., 2011; Davis et al., 2011; Martinez, 2009; Nandi et al., 2004).

PROBIOTICS AND PREBIOTICS

Workshop participants considered two major categories of dietary interventions intended to confer a health benefit: probiotics and prebiotics. To set the stage for discussion on each category of intervention, James Versalovic, head of the Department of Pathology and director of the Texas Children's Microbiome Center at Texas Children's Hospital, provided an overview of probiotics and George Fahey, professor emeritus of animal sciences and Kraft Foods endowed professor emeritus of nutritional sciences

at the University of Illinois, an overview of prebiotics. While there are several potential probiotic mechanisms of action (Neish, 2009; Saulnier et al., 2009), Versalovic elaborated on evidence showing that probiotics can either stimulate or suppress host immunity (Macaubas et al., 2003; Madara, 2004; Prescott et al., 2008; Thomas and Versalovic, 2010; Yamanaka et al., 2003). With respect to host immune suppression, he relayed how his research group made a surprising discovery: the probiotic *Lactobacillus reuteri* can suppress host immunity by secreting histamine (Thomas and Versalovic, 2010; Thomas et al., 2012). "But the real punch line isn't histamine," Versalovic said. "It's histidine." *L. reuteri* bioconverts dietary histidine into histamine. The "other part of the punch line" is that microbially produced histamine suppresses immunity only in the presence of an H2 receptor. In the presence of an H1 receptor, histamine stimulates immunity. He and his team are exploring microbe-derived immunomodulatory molecules. Versalovic speculated that providing enzymatic machinery that converts dietary content into biological signals "may be how the microbiome is really contributing to health and physiology."

In his overview of prebiotics, Fahey summarized the major dietary sources of prebiotics and explored evidence showing how prebiotics selectively stimulate the growth and/or activity of bacteria that contribute to colonic and host health (Davis et al., 2010; Everard et al., 2011; Hooda et al., 2012; Martinez et al., 2010; Mussatto and Mancilha, 2007). While the effect of a prebiotic on the microbiota depends largely on the type of prebiotic ingested and its dietary concentration, Fahey noted that a multitude of other factors affected by the prebiotic will also affect the microbiota, such as intestinal transit time and frequency of defecation. Fahey urged more research on the effect of prebiotics on microbial metabolites, not just the microbiome taxonomic composition.

There are some key scientific challenges to translating probiotic science into probiotic foods, according to Mary Ellen Sanders, executive director of the International Scientific Association for Probiotics and Prebiotics, beginning with the need for a more substantial evidence base that probiotic-mediated changes in the microbiome confer health benefits on the host. That is, there is plentiful evidence that probiotics impact the microbiome and that they benefit human health, but it is not clear whether the observed human health benefits are actually mediated by the microbiome changes (Sanders, 2011). Strain specificity creates another major challenge to interpreting and translating research on probiotics into probiotic-containing food products, with the effectiveness of one strain not necessarily an indication that other strains are equally effective (e.g., see Canani et al., 2007). Yet another challenge is difficulty in demonstrating magnitudes of effect that are meaningful and that make a probiotic intervention worth pursuing. Sanders speculated that the public health significance of demonstrated

small effects may be underestimated. A final challenge discussed was the issue of mixed results from replicative studies. Sometimes, multiple studies on similar end points yield different conclusions about a probiotic's effect. These differences may reflect individual-level variation in microbiome composition or in activity among subjects, or that the study is underpowered (i.e., the sample size is too small to detect an effect). Added to these scientific challenges are regulatory challenges. Sanders expressed concern about draft guidance on when human studies require Investigational New Drug (IND) applications, suggesting that, if finalized, the guidance could have a "chilling" effect on probiotic research in humans.

Experiments carried out within well-controlled laboratory or clinical settings may suggest that a particular bacterium is a highly effective probiotic. Yet if the activity of that probiotic is lost before it reaches a site in the human GI tract where it can exert its beneficial health effects, then that prediction falls flat. David Julian McClements, professor in the Department of Food Science at the University of Massachusetts Amherst, provided an overview of encapsulation technologies that can be used to build delivery systems for probiotics. Embedding a probiotic in some sort of solid or liquid matrix or coating it with some sort of protective layer keeps the probiotic safe (i.e., viable and plentiful) as it travels through the stomach and into the colon (Priya et al., 2011). While most of McClements's research on food delivery systems is with nutraceuticals, he stated that the same systems are amenable to utilization with live bacteria.

Despite the many scientific and other challenges to translating probiotic science into probiotic foods, the food industry already has seen tremendous success. Johan van Hylckama Vlieg, scientific director of gut microbiology and probiotics at Danone Research Center, discussed how Danone is leveraging the microbiome for health with a specific focus on prebiotics and probiotics. He described how microbiome science provides the "rationale" for prebiotic and probiotic interventions. This rationale was illustrated with experimental results from studies on the TRUC mouse model, where the mice were fed a *Bifidobacterium animalis* subsp. *lactis* containing fermented milk product (FMP) (Garrett et al., 2007; Veiga et al., 2010). TRUC mice spontaneously develop gut inflammation resembling human ulcerative colitis. Studies have shown that FMPs decrease gut inflammation in TRUC mice by altering the intestinal environment in a way that inhibits the growth of colitogenic bacteria. In addition to its research on FMPs, Danone is also building a culture collection of microbes, mostly lactic acid bacteria, and identifying strain-specific genes and functions (Diancourt et al., 2007; Siezen et al., 2010), which provides an important resource for future innovation. The strain collection is part of Danone's preparation for what van Hylckama Vlieg predicted will likely bring opportunities to the food industry in com-

ing years: the demand for personalized or categorized nutrition based on individual- or group-level microbiome variations.

UNDERSTANDING CONSUMER BEHAVIOR AND REGULATORY CHALLENGES

Added to the many scientific challenges to realizing the potential of microbiome-targeted dietary intervention as a means to health, speakers also addressed the market and regulatory challenges to realizing that potential. When probiotics were first introduced into the marketplace, consumers were confused, according to Darren Seifer, food and beverage industry analyst for the NPD Group. For example, according to data collected by the NPD Group, in 2006 more adults were trying to cut down on or avoid probiotics (13 percent) than to get more probiotics into their diets (10 percent). Although the trend has shifted, with more adults in 2010 trying to get probiotics into their diets (24 percent) than avoid them (10 percent), there is still some confusion around the word "probiotic." "Prebiotic" is even more difficult. This confusion is just one component of the challenge of changing consumer behavior. Although changing consumer behavior around food is difficult, it can be done. Seifer summarized market research showing that consumers respond to changes that make foods easier to prepare, newness, and the idea of enhancing and not restricting one's diet.

According to Peggy Steele, global business director in the Nutrition and Health Division of DuPont, the probiotic market is one of the fastest-growing sectors in the functional food market. Yogurts account for the majority of new products (75 percent) being launched as probiotics. Over the past several years, the probiotic yogurt market has been growing at about 10 percent annually. The question is, Will that growth persist as the regulatory environment becomes more challenging to maneuver and as manufacturers and marketers are no longer able to make the same type of claims about their products that they have been able to make in the past? Steele suggested three general types of actions that industry can take to help drive continued growth in probiotics in the face of a changing regulatory landscape: (1) conduct efficacy studies to help the scientific and regulatory communities recognize the effects of probiotics on human health (e.g., Ouwehand et al., 2008); (2) educate doctors, nutritionists, key opinion leaders, and journalists to communicate the results of human studies conducted on probiotics; and (3) explore new end points (e.g., new health end points, effects in different populations) (e.g., Amar et al., 2011; Ibrahim et al., 2010; Makelainen et al., 2009).

The changing regulatory landscape around health claims for food products is arguably most visible in the European Union. Seppo Salminen, professor of health biosciences and director of the Functional Foods Fo-

rum at the University of Turku in Finland, described changes that have taken place since the 2006 European Parliament passed new nutrition and health claim legislation. The new regulation creates several challenges for claim applicants, not the least of which is that evidence for an effect must be demonstrated in the generally healthy population (i.e., not a diseased population). In addition to changing the way the European Food Safety Authority (EFSA) evaluates new claims, the 2006 legislation also required EFSA to assess existing nutrition and health claims. With respect to probiotics, this new evaluation involves identifying and characterizing the strain being used, evaluating relevant studies on the proposed health relationship, and assessing whether the proposed health relationship is something that consumers can understand. Salminen commented on the difficulty in characterizing many of the strains being used in currently marketed probiotic products, let alone evaluating whether the evidence supports the proposed health claims. He acknowledged the difficulty in demonstrating a health effect in a generally healthy population but suggested that in many cases, small changes to standardize approaches and outcome measurements in study design would enable researchers to collect relevant data to demonstrate health effects more clearly.

In the United States, a major regulatory challenge for probiotic-containing food products is that many probiotic ingredients require U.S. Food and Drug Administration (FDA) pre-market notification. Dan Levy, microbiologist in the Division of Dietary Supplement Programs at the FDA Center for Food Safety and Applied Nutrition, described draft guidance published in July 2011 to help industry and other stakeholders understand when new dietary ingredient (NDI) notification is necessary and what those notifications should include. He described how FDA evaluates the identity and safety of live microbial ingredients using the same logic it uses to evaluate botanical extracts. Research on the microbiome is advancing so rapidly that it is a challenge to develop specific recommendations.

The health claim regulatory landscape in the United States is governed not only by FDA, but also by the Federal Trade Commission (FTC). Michelle Rusk, senior staff attorney in the Bureau of Consumer Protection at FTC, explained how FDA has primary authority for claims appearing on labeling or product packaging, while FTC has primary authority for claims appearing in advertising (with the exception of prescription drugs, over which FDA has authority over both labeling or packaging and advertising). She described the three steps involved in an FTC investigation, noting that FTC uses the same substantiation standard that FDA adopted in its draft guidance for dietary supplement claims. First, FTC examines the internal validity of the studies that support the claim. Second, it examines the context of the studies that the company is relying on for substantiation (e.g., Are there any inconsistencies and, if so, how are those resolved?). Third,

it examines the relevance of the science to the claim being made. Rusk highlighted two recent FTC actions, one against claims made about two of Dannon's yogurt products, the other against claims made about a Nestlé probiotic drink. She assured the workshop audience that FTC is not raising its standards, but rather is trying to be more transparent and concrete so that companies know exactly what is expected in terms of compliance.

Looking through the lens of DuPont, Stuart Craig, director of regulatory and scientific affairs for DuPont Nutrition and Health, described how the changing regulatory landscape is affecting the food industry. He noted that the EFSA evaluations in particular have drawn on many of DuPont's regulatory affairs resources in the past couple of years. The EFSA evaluations reflect a general global trend toward a higher scientific standard for safety and efficacy with respect to health claims on food products, but the higher standard creates a significant challenge for the food industry. Not only are human clinical studies expensive, threatening return on investment, but also it is more difficult to demonstrate health maintenance than disease intervention. Compounding the challenge is the fact that there is no global system for scientific substantiation. Different regions, sometimes different countries, operate according to their own rules and standards for scientific substantiation, making collaboration and comparison difficult. Craig mentioned some tools that DuPont uses when conducting its own internal scientific substantiation evaluations.

Finally, Sarah Roller, partner with the law firm Kelley Drye & Warren LLP, suggested that many of the regulatory challenges addressed by workshop speakers relate to the fact that "we are struggling to fit" an emerging science into an old legal paradigm. In the United States, the regulatory landscape for health claims on food products was codified in law in 1938, as part of the Federal Food, Drug, and Cosmetic (FD&C) Act. Added to the FD&C Act are the many other federal and, importantly, state laws that govern health claims on food products. Roller explained how an FDA warning letter about a probiotic-containing food product can quickly cascade into a series of state-level actions—namely, class action lawsuits—that have a "chilling" effect not only on the truthful communication of information, but also on industry investment in products that Roller believes have "huge promise for public health." She wondered whether the type of ecological approach that is used in environmental law might be a more useful way to think about food, health, and the microbiome.

MOVING FORWARD

Although research on the microbiome is still widely considered an emerging area of science, the field is progressing quickly. Researchers are making significant headway in understanding not just what the microbi-

ome does, but how the microbiome influences human health and disease, including through its interaction with diet. What we eat and drink influences the microbiome, with significant implications for human health and disease, and the microbiome in turn influences diet. All of this newfound knowledge about diet-microbiome-host dynamics is being used to develop probiotic and prebiotic food products intended to help build and maintain health. Indeed, probiotics are one of the fastest-growing sectors in the global functional food market. Yet, despite this early scientific and market progress, the field faces significant scientific and regulatory challenges. During the last session of the workshop, participants debated ways to move the science forward and drive continued industry investment in microbiome-related product development. Moderator Fergus Clydesdale, distinguished university professor in the Department of Food Science at the University of Massachusetts Amherst, initiated the open discussion by observing that the science of the microbiome is focused mostly on associations between the microbiome and disease, not health, and that most dietary interventions intended to have an impact on host biology via their influence on the microbiome (e.g., probiotics) are being studied for their potential to prevent disease, not promote health. However, current regulatory constraints on food claims prohibit communicating to consumers many of the effects that studies focused on disease prevention demonstrate. Participants debated opportunities for shifting the science by encouraging more research in healthy populations versus shifting the regulatory landscape to accommodate the science. Several suggestions were put forth for how to proceed down each path.

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1

Introduction

Many people think of the skin, or perhaps the lungs, as the principal barrier between human bodies and the outside world. Arguably, it is neither. Many would argue that our most intimate relationship with the outside world is in our gut. Our gastrointestinal (GI) tracts harbor a vast and still largely unexplored microbial world. Microbial cells on or in the human body, not just in the gut but elsewhere as well, outnumber human cells 10 to 1. Scientists are only just beginning to understand what is collectively known as the human microbiome—what it is, what it does, and how it benefits human health. They are recognizing the integral role of the microbiome in human physiology, health, and disease—with microbes playing critical roles in many host metabolic pathways—and the intimate nature of the relationships between, on the one hand, the microbiome and host physiology, and on the other, the microbiome and host diet. While there is still a great deal to learn, especially about the underlying mechanisms driving these relationships, the newfound knowledge already is being used to develop dietary interventions aimed at preventing and modifying disease risk by manipulating the microbiome.

The Food Forum convened a public workshop on February 22-23, 2012, to explore current and emerging knowledge on the human microbiome, its role in human health, its interaction with the diet, and the translation of new research findings into tools and products that improve the nutritional quality of the food supply. The purpose of the workshop was to (1) understand how diet influences the human microbiome, as well as how the microbiome influences the response to diet and dietary components; (2) become familiar with the acquisition of, and potential ways to modify,

the human microbiome to reduce risk and prevent or modify disease; (3) explore the societal and policy implications of applying research findings to the food supply; and (4) identify opportunities for future research and food product and technology development on the interaction between the human microbiome and diet or dietary components and how this interaction influences health outcomes.

This report summarizes the presentations and discussions that took place during the workshop. It summarizes only the statements of participants at the workshop over the course of the two consecutive days. It is not intended to be an exhaustive exploration of the subject matter, nor does it represent the findings, conclusions, or recommendations of a consensus committee process. The goal was to illuminate issues, not resolve them. The workshop served as a mechanism for individuals from a variety of academic, industry, government, marketing research, and other groups to discuss and debate issues openly and to identify possible approaches for addressing some of the more pressing issues pertaining to microbiome-related research and product development.

ORGANIZATION OF THIS REPORT

The organization of this report parallels the organization of the workshop itself (see Appendix A). This introductory chapter sets the stage by summarizing the keynote presentation by Karen Nelson and providing an overview of major workshop themes. Chapter 2 summarizes the presentations and discussion on the wealth of sequencing data that have been accumulating rapidly as a result of advances in sequencing technology. It also covers what researchers have already learned about what microbes inhabit which parts of the body and the trend toward seeking to understand not just what microbes are present, but what those microbes are doing and how their activity influences host health (i.e., session 1). Chapter 3 summarizes the presentations and discussion that focused on associations between the microbiome and health and disease, with a focus on pediatric, oral, and GI tract health and disease (i.e., part of session 2). Toward the end of the first day, speakers began addressing in greater depth not just how the microbiome interacts with its host, but how those interactions are mediated by diet. Chapter 4 summarizes the presentations and discussion that focused on how the microbiome influences host response to diet and dietary components (i.e., parts of sessions 2 and 3). Chapter 5 summarizes the presentations and discussion that focused on how host diet, in turn, impacts the microbiome, the implications of that impact for human health, and the opportunities and scientific challenges to translating this knowledge into tools and products for use in building and maintaining health (i.e., parts of session 3 and all of session 5).

As the workshop progressed, participants began exploring the social and policy challenges, especially around regulation of food claims, to translating all of these new research findings into tools and products for building and maintaining health. Chapter 6 summarizes those presentations and discussions (i.e., session 6). Finally, Chapter 7 summarizes the discussion that took place during the final session of the workshop, when participants were challenged to identify opportunities for future research and product development related to diet-mediated interactions between the microbiome and human health.

KEYNOTE ADDRESS: THE FUTURE IMPACT OF BENEFICIAL MICROBES AND GUT HEALTH[1]

Microbial cells that populate the human body outnumber human cells by an order of magnitude, with the most densely populated areas being the nasal, oral, skin, gastrointestinal, and urogenital environments. Scientists are only just beginning to understand what these microbes do, how they function, and how they can be manipulated to benefit human health. Research on the human microbiome has benefited tremendously from other recent advances in microbiology, not the least of which is a growing recognition of the vast microbial diversity that exists. Keynote speaker Karen Nelson mentioned Craig Venter and colleagues' circumnavigations of the globe to collect seawater samples and study oceanic microbial diversity (Rusch et al., 2007; Venter et al., 2004; Wu et al., 2011; Yooseph et al., 2007). According to Nelson, that work led to a doubling of the number of predictive protein signatures[2] "essentially overnight." The lesson learned, she said, was "that there is a tremendous amount of microbial diversity in the environment that we have not tapped … we really don't know how much diversity is out there."

Advances in Sequencing Technologies

In addition to spawning a realization of how vast the microbial world is, studies of microbial diversity in other (non–human body) environments also helped the development of advanced sequencing technologies that are now driving research on the microbiome. Nelson recalled how exciting it was when she and colleagues (Eckburg et al., 2005) used Sanger sequencing of the 16S ribosomal RNA (rRNA) gene to evaluate microbial diversity in six major subdivisions of the GI tract even though they were unable to interpret the significance of their results at the time. Shortly thereafter,

[1] This section summarizes Karen Nelson's keynote presentation.

[2] Nucleotide sequence signatures that indicate the presence of a particular protein.

Gill et al. (2006) conducted the first metagenomic study of the human gut microbiome. Metagenomics describes the ability to sequence all the genetic material in a sample without initially having to cultivate the microbial species that are present. The development of these methods was a huge step forward over sequencing methods that were focused on a single phylogenetic marker (as was the case with 16S rRNA) and other methods that were dependent on a PCR (polymerase chain reaction) amplification step that is now known to potentially introduce significant bias. For the Gill et al. (2006) study, even though the researchers sampled from only two individuals, all that microbial diversity again elicited much excitement. At that time, the J. Craig Venter Institute (JCVI) had one of the largest sequencing centers in the world, running about 100 Sanger sequencing machines around the clock that generated about 1-2 megabases per day. Today, the newest high-throughput sequencing technology is capable of generating an entire human genome in about 4 hours.[3] Nelson noted that her first microbial genome sequence project involving the genome of Thermotoga maritime took approximately 2 years and cost about $2 million. Today, the same project could probably be done in an afternoon for less than $200. She predicted that sequencing technology will continue to advance. "I believe that sequencing is going to become like PCR," she said. "Every grad student is going to have their own sequencing machine on their desktop."

The largest human microbiome sequencing study to date is the National Institutes of Health (NIH)-funded Human Microbiome Project (HMP), whose focus is on generating a metagenomic reference database for "normal" individuals to serve as a resource for researchers studying microbiome-disease associations and other phenomena. The reference dataset is based on a human cohort of 300 individuals, with microbial genome data being collected from five major body sites (nasal, oral, skin, GI, and urogenital environments). When the project started, the goal was to sequence 1,000 reference genomes. Today, the goal is to sequence 3,000 reference genomes. Results from the first 178 genomes sequenced were published in 2010 (Human Microbiome Jumpstart Reference Strains Consortium, 2010). In addition to its mostly bacteria-focused work, the HMP also has an initiative to sequence several viruses and microeukaryotes that are associated with the human body.

The Microbiome and Disease

In parallel to the HMP, a number of other organizations have been funding microbiome work focused on specific diseases, with many researchers taking systems biology approaches and integrating multiple -omics

[3] The human genome contains an estimated 3,000 megabases.

technologies (e.g., transcriptomics, proteomics, glycomics, metabolomics). Nelson perceives the field as moving away from "just sequencing" toward "integrating all these different -omics approaches."

In addition to its involvement with the HMP, JCVI itself has about 20 disease-focused metagenomic studies funded not just by NIH but also by the National Science Foundation (NSF), the Bill & Melinda Gates Foundation, the National Aeronautics and Space Administration, and others. For example, the National Institute of Diabetes and Digestive and Kidney Diseases recently awarded JCVI a $5 million grant to study the gut microbiome and virome, along with the urinary proteome and metabolome, in an effort to identify a panel of biomarker candidates for type 1 diabetes. JCVI will be recruiting children with type 1 diabetes and using their healthy siblings as controls. NIH also funded JCVI in collaboration with researchers from New York University to examine how the microbiome and virome change over time in individuals with esophageal cancer. Investigators are following 80 individuals over 4 years; the study is currently entering its final year. Already they have detected microbial signatures associated with different stages of esophageal cancer. It is unclear whether the microbial changes are causing the cancer or the cancer is causing the microbial changes. Either way, Nelson said, "You can imagine new therapies that are based on … restoring what the normal [microbial] population looks like." A third example of disease-focused JCVI research is a study being conducted in collaboration with Dr. David Brenner at the University of California, San Diego, on liver damage and alcoholism. Using different mouse models, the researchers have demonstrated a correlation between certain changes in microbial metabolites and disease onset (Fouts et al., 2012; Yan et al., 2011).

The Health and Wellness Potential of Microbiome Manipulation

Also at JCVI, Dr. Roger Lasken has created what Nelson described as a "high-throughput pipeline" for generating genomes of microbial species that cannot be cultivated. The methodology is based on cell sorting mechanisms and multiple displacement amplification (MDA). Nelson noted that this type of nontraditional approach is necessary for accessing genomes of the 98 to 99 percent of microbes that cannot be cultivated. As scientists learn more about the role of the microbiome in human health and wellness, accessing that genomic space will become increasingly desirable. Nelson foresees this once-inaccessible genomic information being used to develop novel therapeutic and nutritional (e.g., probiotic) tools in the future.

Yet before the health and wellness potential of microbiome manipulation can be realized, the field faces some key challenges. Nelson identified informatics as one of the "big gaps." She said, "I think we are getting ahead of ourselves in terms of being able to interpret the data that are be-

ing generated." Another key challenge is the lack of communication and collaboration among experts in microbiology, nutrition, and other relevant fields. She encouraged more dialogue across disciplinary groups and more industry participation in the dialogue.

Challenges with Experimental Design

During the question-and-answer period following Nelson's talk, most of the discussion revolved around experimental design, including tissue sampling, sample size, and the definition of "normal." An audience member suggested that sampling from the small intestine would be more informative than sampling from fecal samples or from the esophagus. Nelson agreed that the field needs to move in a direction where scientists are sampling from other parts of the GI tract, but researchers have done as well as they have been able in the early stages. Sampling from the small intestine would be more invasive than fecal sampling. She suggested that more cross-disciplinary dialogue, in this case with medical researchers, could help move the field in that direction. Another question was raised about the value of a study based on a sample size of 300, let alone 2, individuals (the person asking the question was referring to Nelson's mention of the $N = 300$ sample size of the HMP reference database and the $N = 2$ sample size of the Gill et al., 2006, study). Nelson agreed that the field needs to move in that direction, that is, toward sampling large host populations, but again she said, "We did what we could do at that point in time." Another audience member asked how HMP investigators define "normal." Lita Proctor agreed that the question is "nontrivial." Defining normal was a struggle. They finally decided that for the purpose of the HMP, "normal," or "healthy," meant verification of no overt disease based on clinical examination.

MAJOR OVERARCHING THEMES

The microbiome is integral to human physiology, health, and disease.

- Scientists are beginning to recognize the microbiome as an extra level of biological complexity that is integral to human physiology, health, and disease. Some workshop participants perceived the microbiome as an extension of human metabolism, with gut bacteria playing key roles in host metabolism. Native bacteria impact not only which dietary components their host is able to extract from its diet, but also how those dietary components are converted into biological signals. Gut microbes also impact host energetics. Neither chronic nor infectious disease risk can be understood without also taking into account the microbiome. Indeed, the microbiome is increasingly being

viewed as a target for diagnostic, prognostic, and even therapeutic approaches to predicting or managing various disease conditions.
- As much as scientists are learning about associations between the microbiome and physiology, health, and disease, the microbial world inside us remains a vast and largely untapped world. As Peter Turnbaugh asked, "What additional functions or metabolic capabilities are provided us by these communities, and how does that impact our health and disease?"

The microbiome is arguably the most intimate connection that humans have with their external environment, mostly through diet.

- A major recurring theme of the workshop discussion was the very intimate connection that the human microbiome has with both its human host and its host's external environment. Diet appears to be the most important environmental modulator of the microbiome, with significant implications for human health and disease. As scientists continue to learn about the impact of diet on the microbiome and the consequences of that impact for human health and disease, the food industry is using that newfound knowledge to develop novel products for building and maintaining health via their impact on the microbiome.

Given the emerging nature of research on the microbiome, some important methodological issues still have to be resolved with respect to undersampling (i.e., some workshop participants expressed concern not just about underpowered studies, but also about tissue undersampling) and a lack of causal and mechanistic studies.

- In almost every open discussion, individual workshop participants or audience members expressed concern about the danger of making predictions about diet-microbiome-health relationships based on studies with small samples sizes. For example, Ellen Silbergeld said, "I think the comments that have been raised throughout this meeting about how we're really dealing with a very small edge of knowledge when we talk about the microbiome in any specific domain should give us pause when we make predictions as to what is going to happen." She remarked that small studies are helpful for formulating new hypotheses, but they are not sufficient for translating science into public health. She and others cautioned that "large-*N*" studies will be needed in the future. Johan van Hylckama Vlieg and other participants agreed that more large-*N* studies are needed but emphasized that small-*N* studies serve an essential exploratory

role, enabling researchers to generate testable hypotheses for those larger studies. In addition, well-designed and small sample studies can contribute to building a mechanistic understanding of clinical observations obtained in larger studies.

- With respect to fecal sampling, participants expressed concern that making inferences about what is happening inside the gut based on what is detected in feces can be dangerous given that the microbiome is a dynamic, complex system that is highly individual and easily perturbed. Jeremy Nicholson said, "For me, it is like trying to sniff an exhaust pipe of a Ferrari and tell you what color [the car] is. You have this very complex ecology which you have compressed into a piece of feces.… I think we need to develop new technologies to be able to study the microbes in situ and what they are doing locally." Again, however, given its noninvasive nature, fecal sampling has been the only choice in many of these early studies.
- There were some calls for more mechanistic research. Even when sequencing data are complemented with functional annotation, purported functions are just that—purported. They still need to be validated with mechanistic study—thus, the importance of animal models or even non-animal models.
- Some workshop participants also called for more longitudinal studies as a way to examine causality. Much of what is being learned about diet-microbiome-health relationships is correlational, not causational (e.g., that a particular microbial strain or microbial metabolite is associated with a disease risk, but with no clear understanding of which came first).

Dietary interventions intended to have an impact on host biology via their impact on the microbiome are being developed, and the market for those products is seeing tremendous success. However, the current regulatory framework threatens to slow industry interest and investment.

- Much of this early research on the microbiome focuses on associations between the microbiome and disease, not health, and most dietary interventions intended to have an impact on host biology via their influence on the microbiome (e.g., probiotics) are being studied for their potential to prevent disease, not promote health. However, current regulatory constraints on food claims prohibit communicating to consumers many of the effects that studies focused on disease prevention demonstrate. Some workshop participants noted the challenges and value of conducting more studies in healthy populations versus changing the regulatory landscape to accommodate the science.

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2

Study of the Human Microbiome

While study of what is now known as the human microbiome can be traced as far back as Antonie van Leeuwenhoek (1632-1723), advances in genomics and other areas of microbiology have spurred a resurgence of interest. Much of this interest has been driven by and directed toward genomics, with a major goal of the Human Microbiome Project (HMP) being to characterize the genomic makeup of all microbes inhabiting the human body. However, increasingly, scientists are shifting their attention toward studying not just what microbes are present, but what those microbes are doing. This chapter summarizes the workshop presentations and discussion that revolved around some of this early (contemporary) scientific research on microbiome content and function.

DEFINING THE HUMAN MICROBIOME[1]

> *I then most always saw, with great wonder, that in the said matter there were many very little living animalcules, very prettily a-moving.* —Antonie van Leeuwenhoek (1632-1723)

While there is no doubt that microbes create some of the world's greatest disease challenges (malaria, cholera, foodborne illness, and other infectious diseases), in fact 99 percent of microbes do not cause disease. There are many beneficial microbes that contribute to food production (e.g., the

[1] This section summarizes Lita Proctor's presentation.

production of bread, cheese, yogurt, chocolate, coffee, beer); soil production and regeneration; pollutant and toxin degradation; oxygen production; and plant, animal, and human health. Lita Proctor remarked, "Every living thing on this planet has a microbiome … associated microbes that maintain health and well-being." She defined the microbiome as the full complement of microbes (bacteria, viruses including bacteriophages, fungi, protozoa) and their genes and genomes in or on the human body.

That there are beneficial microbes living in and on the human body is not a new concept. Proctor traced the notion as far back as van Leeuwenhoek. "Four centuries ago," she said, "we realized that there are lots of microbes associated with our bodies. But it has taken four centuries for us to really look at these microbial communities in any depth and to consider them not just as pathogens." While advances in sequencing and other technologies are no doubt contributing to this burgeoning research, Proctor acknowledged the significant contributions of other scientific disciplines. Notably, environmental microbiology and microbial ecology and evolution "really set the conceptual framework for … recognition that the vast majority of microbes that live in and on us are not germs or pathogens but belong there and actually help maintain our health and well-being."

The Human Microbiome Project

The HMP was initiated by the National Institutes of Health (NIH) in the fall of 2007, with the majority of funding ($153 million of the $173 million to date[2]) coming from the NIH Common Fund. The Common Fund is designed to catalyze new and emerging areas of science. The HMP used sequencing to examine the microbes associated with the human body. Its main purpose is to create resources for the research community, with a focus on building a "healthy cohort" reference database of human microbiome genome sequences (known as metagenomic sequences), computational tools to analyze complex metagenomic sequences, and clinical protocols for sampling the human microbiome. Other resources include the suite of demonstration projects that provide data on the association of microbiomes with disease. The "healthy cohort" project is a sequencing study of the microbiome based on sampling from 5 major body sites (18 subsites): nasal passages, oral cavities, skin, gastrointestinal (GI) tract, and urogenital tract. The body sites were selected by a panel of experts in human microbiology. The study recruited 300 adults (of whom half were women and half were men) who were clinically verified to be free of overt disease. About 20 percent of the study participants self-identified as a racial minority and 10 percent as Hispanic. Each participant was sampled up to three times

[2] As of February 2012 (i.e., at the time of the workshop).

over a 2-year period. Two kinds of sequencing data were collected: microbial taxonomic characterization using the 16S ribosomal ribonucleic acid (rRNA) marker gene and sequence data from entire microbial communities (i.e., metagenomic sequences).[3]

In addition to the healthy cohort project, the HMP is managing a series of demonstration projects to evaluate associations between the microbiome and disease: two skin diseases (eczema and psoriasis), five GI tract diseases (Crohn's disease, esophageal adenocarcinoma, necrotizing enterocolitis, pediatric inflammatory bowel disease [IBD], and ulcerative colitis), and four urogenital conditions (bacterial vaginosis, circumcision, reproductive history, and sexual history).

Additionally, the project is accumulating clinical and phenotype data associated with either the healthy cohort sequencing data or sequencing data from the demonstration projects and is planning to collect nucleic acid extracts and, potentially, cell lines from the healthy cohort. All of the various "moving parts" of the HMP interact through the Data Analysis and Coordination Center and the 200-plus member HMP Consortium.[4] Also, the HMP is a founding member of the International Human Microbiome Consortium (IHMC).[5]

One of the limitations of the HMP is its exclusion of host genetic data. One reason that host genetic data were not collected was subject consent (i.e., subjects participating in the various studies agreed to public release of only certain types of data). Proctor called attention to a 2011 article (Spor et al., 2011) for a review of the scientific literature on the putative relationship of host genetics with the microbiome.

Universal and Personalized Properties of the Human Microbiome

HMP and other recent research on the microbiome have generated plentiful new knowledge, enough to begin to identify "universal" properties of the microbiome. Proctor listed several. First, the human microbiota is acquired anew each generation, at birth. Proctor described newborns as "microbe magnets." Dominguez-Bello et al. (2010) reported that babies born vaginally acquire a different microbiome than babies born via cesarean section (C-section), with the primary inoculum for vaginally born babies being the mother's vaginal microbiome and for babies born via

[3] See the next section in this chapter, a summary of Jennifer Russo Wortman's presentation, for a detailed explanation of how the two different types of data were analyzed and interpreted.

[4] Visit the HMP website for more details: commonfund.nih.gov/hmp (accessed August 28, 2012).

[5] For more information on the IHMC, visit its website: www.human-microbiome.org (accessed August 28, 2012).

C-section, the mother's skin or the environment. The fact that the microbiome is acquired anew each generation is in stark contrast to the human genome, which is inherited.[6]

A second universal property is that each adult body part has a distinct microbial community composition. HMP 16S rRNA data reveal a clustering of certain microbial taxa with particular body sites, such as the skin, gut, oral cavity, airways, or urogenital tract, regardless of host gender, age, weight, or any other host metric. Costello et al. (2009) reported a similar finding—that microbial community composition is dictated by body site. Proctor observed that body site clustering is probably driven by the same types of factors that drive microbial colonization and growth in other environments (i.e., pH, temperature, condition of the substrate, other ecological parameters). She said, "The human microbiome is probably like a lot of other microbial ecosystems out there on the planet."

However, while 16S rRNA data show that microbial composition varies among body sites and even within body sites between individuals, metagenomic data indicate that the major microbial metabolic pathways are effectively the same across body sites. So even though each body site has its own unique microbial assemblage, all of those assemblages, regardless of composition, appear to function similarly with respect to metabolism. This is true for healthy individuals in the HMP study, but it remains to be seen how microbial metabolism compares between healthy and diseased individuals.

A final universal property of the human microbiome is that the gut microbiome changes over a lifetime, with microbiomes in elderly people (aged 65 and over) being very different from microbiomes in middle-aged adults. As part of the ELDERMET project,[7] Claesson et al. (2011) reported a greater proportion of Bacteroidetes, more overall microbial taxonomic diversity, and greater individual variation in microbial taxonomic composition among elderly compared to middle-aged individuals. With babies, microbial succession during the first 1 to 2 years of life begins to vary with the transition to a more diverse diet (Yatsunenko et al., 2012), as opposed to the relative stability seen with breast-fed infants. Eventually, by the second year of life, the taxonomic composition of the gut microbiome stabilizes, and the gut develops what appears to be an adult microbiome (Palmer et al., 2007).

On the basis of HMP studies, Proctor noted that evidence to date does not support the notion of a core microbiome, at least not at the species level; the concept of enterotypes; or the classification of microbiomes of

[6] See the summary of Josef Neu's presentation in Chapter 3 for a discussion of microbiota acquired prior to birth, during the third trimester of pregnancy.

[7] Funded through the government of Ireland, ELDERMET is a study of diet, gut bacteria, and health status in elderly (65 years and older) Irish subjects.

any one body site into distinct subsets. None of those properties, in her opinion, is universal. With respect to the notion of a core microbiome, although reproducible subsets of microbes may be found in all individuals at grosser taxonomic levels, such as the phylum level (Backhed et al., 2005) and perhaps at the genus level for some body sites (e.g., the skin and possibly the vaginal microbiomes), Proctor questioned the validity of the notion at the species level. In fact, the finer the taxonomic classification, the more variable the microbial composition is among individuals. She posed the question, Can humans be grouped by enterotype? HMP and other data suggest that the question is still open (Wu et al., 2011). "It is a very attractive concept, and it could still play out," Proctor said, "but it is not showing up as a reproducible universal property of microbiomes, in our opinion." Finally, with respect to the notion that a healthy microbiome is defined by the absence of pathogens, she noted that HMP healthy cohort data showed that the sequences of putative pathogens are in fact present in healthy individuals (Zhou et al., unpublished manuscript). The presence of a pathogen sequence does not necessarily mean that the pathogen is actually playing a pathogenic role. Often, it is not the presence of a pathogen that indicates disease, but rather an imbalance in the microbial ecosystem.[8]

On the basis of the HMP data, Proctor views the microbiome as a personalized property. The vast majority of taxonomic diversity in the microbiome is at the species and strain levels, with the abundance of any one bacterial species varying by up to four orders of magnitude between individuals (Backhed et al., 2005; Qin et al., 2010). In Proctor's opinion, we each have our own "personal microbes" that "confer particular properties to each one of us," but it's not yet clear what those properties are.

The Virome

While most of the workshop presentations and discussion focused on the bacterial components of the human microbiome, Proctor reminded the workshop attendees of the vast viral world that inhabits the human body. In fact, there are an estimated 10 times more virus-like particles than bacteria in and on the human body. The human "virome" includes bacteriophages, eukaryotic viruses, and endogenous viral elements. Bacteriophage diversity in the human microbiome is greater than in other environments (i.e., mosquito, coral reef, human lung, and free-living environments) (Caporaso et al., 2010).

[8] For two additional perspectives on the role of an out-of-balance microbiome in human disease, see the summaries of presentations by Richard Darveau and Vincent Young in Chapter 3.

Very Close Association Between the Human Microbiome and Our Environment

A major overarching theme of the workshop was the very close association that exists between the human microbiome and our external environment. Proctor highlighted three phenomena that reflect this close association over very different timescales: (1) the impact of antibiotics on the microbiome, (2) the high rate of horizontal gene transfer between bacteria in the microbiome and bacteria in the environment, and (3) changes in the microbiome over evolutionary time.

Antibiotic exposure has tremendous consequences for the microbiome. Proctor relayed Jernberg et al.'s (2010) description of the impact of antibiotics on the microbiome and the cascade of events that occur when an individual stops antibiotic treatment. First, antibiotic-resistant microbes increase in number. Second, susceptible bacteria, that is, bacteria that could have been killed by the antibiotic but were not because they picked up resistant genes through horizontal gene transfer with their neighbors, increase in number. Third, bacteria that were never actually exposed to the antibiotic because they were embedded in mucus or otherwise protected from exposure increase in number. The result is an overall increase of resistant and protected bacteria. "We can often cause more problems than we cure in many cases when we take antibiotics," Proctor said, "especially when we don't take the full regimen."

Horizontal gene transfer (the exchange of genes between microbes in the absence of sexual reproduction) between bacteria in the microbiome and bacteria in the environment also has tremendous consequences for the microbiome. Smillie et al. (2011) calculated rates of horizontal gene transfer among more than 2,000 bacterial genomes and reported a greater frequency of horizontal gene transfer in the human microbiome than in other environments, with the most transfer occurring among microbes inhabiting the same body sites (e.g., the microbes of two gut microbial communities are more likely to engage in horizontal gene transfer than a gut microbe and a skin microbe). The researchers concluded that horizontal gene transfer is being driven not by physical proximity, but rather by ecology. Importantly, they also reported that the highest rates of horizontal gene transfer between human-associated and nonhuman microbes were with farm animal microbes.[9]

Finally, in Proctor's opinion, understanding the evolutionary context of the microbiome sheds light on the "ultimate connection" between the

[9] For an in-depth discussion of horizontal gene transfer and its role in spreading antibiotic resistance from livestock farms to the human microbiome, see the Chapter 5 summary of Ellen Silbergeld's presentation.

human microbiome and our external environment. Proctor speculated on the evolutionary history of a group of human immune genes, the human leukocyte antigens, which appear to be derived from other early hominids that interbred with *Homo sapiens* (Abi-Rached et al., 2011). Proctor said, "It is not really a stretch to also suggest that not only were genes shared, but also the microbiome was shared." By deriving some of its microbiome from other early hominids, *H. sapiens* may have been better equipped to deal with novel infectious diseases and other stressors as it migrated out of Africa and into new environments. Several scientists have suggested that contemporary societal practices (e.g., sanitation, clean water, bathing, antibiotic use, cesarean birth, formula feeding, mercury amalgams) are creating an environment in which humans' microbiomes are no longer exposed to the rich diversity of microbes they used to be exposed to in our evolutionary past. Blaser and Falkow (2009) suggested that if the initial inoculum is coming from the mother, but every next generation of mothers is more microbially impoverished than the previous generation, then fewer and fewer beneficial microbes are being acquired every next generation.

Mutability of the Microbiome: Proposed Microbiome Therapeutics and Diagnostics

NIH is currently examining the possibility of a next phase of this project. However, the HMP is not the only microbiome-related NIH investment. According to Proctor, the rate of funding for microbiome research is accelerating across all of the various NIH institutes. Some of this funding is for applied research on microbiome therapeutics and diagnostics. Proctor listed five categories of proposed therapeutics and diagnostics:

1. The use of microbiome signatures as biomarkers for disease presence;
2. The use of enterotypes to classify individuals by disease risk or pharmacokinetics;
3. The use of antibacterials, anti-inflammatories, and other small molecules produced by microbiome community members for therapeutic purposes;
4. The engineering of novel microbiome strains to stimulate T-cells, produce pro-inflammatory cytokines, stimulate expression of host antimicrobial factors, deliver drugs, and so forth; and
5. The development of virome biomarkers or other tools that exploit the virome for therapeutic or diagnostic purposes.

TOOLS AND MODELS FOR ASSESSMENT OF THE MICROBIOME[10]

HMP investigators are collecting three types of microbiome sequencing data: 16S rRNA sequences,[11] shotgun sequences,[12] and reference genome sequences.[13] Jennifer Russo Wortman described how HMP Consortium investigators are using this sequencing data to address three key questions: (1) What organisms are present? (2) What do they do? and (3) How do they change in health and disease?

Wortman referred workshop participants to a review article (Kuczynski et al., 2012) for more detail on some of the methodologies she covered (for more information on caveats of the different sequencing technologies, informatics challenges, etc.).

Phylogenetic Analysis: Who Is There?

Investigators are using 16S rRNA sequencing data to address the question, What organisms are present? The initial HMP analysis yielded about 72 million 16s rRNA reads. As Wortman said, "That is a lot of sequenced data to analyze." The goal was to use those 72 million reads to get a sense of not only which species are present in the various body sites, but also how abundant the various species are. Very generally, using various quality controls, "de-noising" algorithms, and other computational tools (Caporaso et al., 2010), the 72 million reads were clustered into what are known as operational taxonomic units (OTUs). OTUs are proxies for species. OTU data can be used not only to identify how many of which species are present (per-sample OTU counts), but also to infer the evolutionary relationships of those present.

There are two ways to classify OTUs. The first is to use what is already known about 16S rRNA sequences from cultured organisms, that is, data already stored in various reference databases (e.g., the RNA Database Project, or RDP). By comparing 16S rRNA sequences from HMP samples to those reference sequences, in most cases researchers can identify their samples to at least the family or genus level. Refer-

[10] This section summarizes Jennifer Russo Wortman's presentation.

[11] The 16S rRNA gene encodes for a small subunit of the ribosomal RNA. HMP researchers use 16S rRNA sequencing for phylogenetic analysis because the gene has both conserved regions (which are used to develop primers for amplification) and variable regions (which are used to identify specific microbial species).

[12] HMP researchers use shotgun sequencing to sequence all of the DNA that is present within a microbial community. By comparing specific sequence reads to sequences with known functions, they can infer function.

[13] HMP researchers are sequencing as many microbiome reference genomes as possible as part of the "healthy cohort" study that Lita Proctor described (see previous section for a summary of her presentation).

ence database comparisons are limited by the fact that not all sampled sequences are covered in these databases, and species-level assignments in particular are hard to find; however, because the method yields very little noise, researchers can be fairly confident of the assignments that are made. The second method is de novo clustering, that is, clustering 16S rRNA sequences on the basis of similarity in sequence (e.g., by allowing only up to 3 percent divergence). De novo clustering yields more granularity, that is, more species-level assignments, but it also generates more noise (e.g., sequencing and amplification artifacts). Because of the different advantages and disadvantages of each method, HMP investigators use both methods to analyze HMP data.

As an example of how OTU classification is being used to analyze the presence and abundance of microbes, HMP researchers analyzed the presence and abundance of bacterial species in stool samples from 200 subjects. While the presence of specific genera was relatively constant among individuals, the relative proportions of those genera were extremely variable. As another, non-HMP example, Wortman mentioned the Kostic et al. (2012) study of the colorectal cancer microbiome. The researchers detected a very clear signal that people with colorectal cancer have an enrichment of *Fusobacteria* in their tumor tissue. As a final example, HMP researchers used both reference-based and de novo OTU classification to analyze OTU data from all five major body sites among the "healthy cohort" study individuals. Reference-based OTU classification methods were used to analyze genus-level trends, while de novo classification was used to analyze species-level trends. Results of the two methods were consistent for all body sites except for vaginal samples, where researchers found the least amount of genus-level diversity but high levels of species diversity. According to Wortman, previous work by Jacques Ravel and colleagues (2011) has shown that the vaginal microbiome is dominated by the *Lactobacillus* genus but that there are many different *Lactobactilli* species present in various abundances.

Metabolic Reconstruction: What Are They Doing?

The goal of metabolic reconstruction is to identify putative pathways by assigning enzymatic functions to sequencing reads wherever possible, based on information in various enzymatic functional databases. As with the phylogenetic analysis, HMP researchers started with a large volume of data, in this case about 3.6 terabases of shotgun sequencing data from 690 samples. Again, they examined both presence (Which pathways are present?) and abundance (How much of each pathway is present?). They used a software program, the HMP Unified Metabolic Analysis Network

(HUMAnN),[14] to link the reads to known enzymatic functions and putative pathways.

Wortman echoed what Lita Proctor had emphasized about compositional diversity being greater than functional diversity with respect to variation among different individuals, based on a comparison of phylogenetic analysis and metabolic reconstruction results. In other words, there is a lot more variation in phylotypes than in pathways. "If there is such a thing as a core microbiome," Wortman said, "it may be at the level of function more than at the level of organism."

Challenges

Wortman identified five major challenges to HMP data interpretation:

1. Meeting data volume and computational requirements. Reiterating what Proctor said, Wortman urged development of an infrastructure for people to access available data. She also cited a need to increase algorithm efficiency and reduce data redundancy.
2. Linking microbiome function to community composition. How can the two different types of analyses (phylogenetic analysis and metabolic reconstruction) be linked so that more nuanced questions can be addressed? (In other words, Which organisms are responsible for which functions?)
3. Integrating different types of -omics datasets. All of the shotgun sequencing data are genomic-level data, with very little functional validation that the identified pathways are active. How can genomic data be integrated with transcriptomic, proteomic, and metabolomic data integrated into a systems biology–level approach to studying these communities?
4. Modeling microbiome community dynamics. How do microbiomes change over time? What are the drivers of those changes? The environment? Health status? How does the prevalence of certain species affect other species in the community?
5. Correlating microbiome shifts with host phenotype. It can be very difficult to associate shifts in community composition (or functional state) with host phenotype when the phenotype in question is not well defined and when the impact of environmental change on that phenotype is unknown.

[14] For more information, visit huttenhower.sph.harvard.edu/humann.

METABOLOME AND MICROBIOME[15]

Personalized health care solutions demand an integrative, systems-level approach to the understanding of human biocomplexity. Research on the microbiome is a core component of that approach (Nicholson, 2006) (Box 2-1). Genes are just one component of the gene-diet-microbial interactions that make humans the "super system" they are. So while genome-wide association studies (GWAS), for example, are very popular, they are not always, in Jeremy Nicholson's words, "tremendously revealing," because statistical significance often has very little to do with biological significance. Speliotes et al. (2010) reported having found 32 statistically significant body mass index (BMI)-associated genes; in fact, the BMI-linked genes accounted for only 1.47 percent of the variance in BMI. Nicholson said, "It is all the other stuff in the world that is really important with the BMI." He mentioned work by Jeffrey Gordon and colleagues showing a connection between obesity and the microbiome (Ley et al., 2006; Turnbaugh et al., 2006). While the obesity-microbiome connection is controversial, Nicholson pointed out that the mixed results driving the controversy are due to differences in levels of phylogeny and in the way the experiments have been conducted.[16]

This is not to say that systems-level studies of human genome complexity are not generating interesting information. They are. For example, Loscalzo et al. (2007) used a systems-level approach to show that almost all human diseases are genetically connected, with the same gene(s) being implicated in different disorders. However, understanding the human genome itself is not enough if the vision of personalized, or "precision," health care is to be realized (Mirnezami et al., 2012). The microbiome represents yet another entire level of genetic connectivity. The challenge for the future, Nicholson said, is "to think about layers of networks on top of networks." That is, how does the human genome network(s) interact with the microbiome genome network(s), across both time and space? "This is really quite a tough problem," Nicholson said, "probably the toughest problem in 21st-century biology."

The Metabolic Window on Complex System Activity

Complex interactions between the microbiome and its host generate more than differential disease risks. They also generate differential metabolic phenotypes (Holmes et al., 2008a). In fact, disease risks and metabolic phenotypes are both biologically and statistically linked such that one can

[15] This section summarizes Jeremy Nicholson's presentation.

[16] See the summary of Peter Turnbaugh's presentation in Chapter 3 for a more detailed discussion of evidence suggesting an obesity-microbiome connection.

BOX 2-1
Research on the Microbiome: Major Research Questions

Understanding human biology and the complex interactions that determine individual and population phenotypes, including disease risk factors and etiopathogenesis, demands a systems-level approach, Jeremy Nicholson asserted. Understanding the microbiome is a core component of that approach:

- Who is there? That is, what microbes are present? What genes are present? The gut microbiome alone contains an estimated 3.3 million genes, but scientists know very little about what most of those genes do, or how they interact (Qin et al., 2010).
- How did they get there? The microbiome is a dynamic system, with gut microbes regularly swapping genes with environmental microbes via horizontal gene transfer. For example, Hehemann et al. (2010) reported that gut microbial metagenomes in Japanese individuals code for seaweed-digesting enzymes that the gut microbial metagenomes of North American individuals do not encode. It is likely that the Japanese individuals acquired the microbial genes from marine bacteria associated with nori. Nori is seaweed traditionally used to prepare sushi, which is a daily component of the Japanese diet. (See the summary of Ellen Silbergeld's presentation in Chapter 5 for a more detailed discussion of the impact of horizontal gene transfer on the microbiome.)
- What are they doing (besides digesting seaweed)? In Nicholson's opinion, metabolic profiling via reconstruction of genomic readouts indicates only what the microbes could be doing, not what they actually are doing.
- How are they doing whatever it is they are doing? For example, how are they communicating? What metabolic and immunological signals are they sending?
- How does what they are doing impact the host?
- Finally, how can all of this knowledge about the microbiome be made into something useful? How can the microbiome be manipulated for health, for example, through diet?

SOURCE: Jeremy Nicholson's workshop presentation, February 22, 2012.

assess disease risk by measuring metabolite levels. Metabolite analysis falls under the purview of what Nicholson calls "metabonomics," which he defined as "the quantitative measurement of the multi-parametric (time-related) metabolic responses of complex systems to a patho-physiological stimulus or genetic modification" (Nicholson et al., 1999). Some scientists, such as Fiehn (2002), use a slightly different term: "metabolomics."

Regardless of terminology, the idea that changes in metabolic products are an indication of disease is not new. For example, urine wheels were

used in the 16th century to diagnose and treat disease (based on the color, smell, and taste of urine). Today, scientists use advanced metabolic profiling tools, namely nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry, that yield a tremendous amount of complex data—a single run generates data on hundreds or thousands of molecules. Researchers use various mathematical modeling tools to extract and convert relevant data into biologically useful information (e.g., information that can be used to identify "normal" versus "abnormal" metabolism). The complexity of the information generated by advanced metabolic profiles is due to the fact that not only are all human cells producing metabolites (with more than 500 functionally distinct cell types), but so too are all microbial cells (Nicholson et al., 2005). Microbes produce short-chain fatty acids, bile acids and related oxysterols, vasoactive (aromatic) amines, cresols and aromatic acids, endocannabinoids, and other molecules.

Many microbial metabolites participate in human metabolism in what Nicholson referred to as "combinatorial metabolism." For example, bile acids, which are critically important host signaling molecules, are co-metabolized by microbes, with significant implications for liver and colonic disease risk (Nicholson and Wilson, 2003). Bile acids are synthesized in the liver on a daily basis and then secreted into the mammalian gut, where they are deconjugated into cholic acid by *Lactobacillus* and other gut microbiota. The cholic acid, in turn, can be dehydroxylated by yet other microbes into deoxycholic acid. Deoxycholic acid is both hepatoxic and carcinogenic. Microbial co-metabolism of bile acids also impacts lipid bioavailability.

Modifying Host-Microbiome Metabolic Interactions: Mouse and Rat Models

When thinking about how the gut microbiome impacts human metabolism, the tendency is to think about distal colon processing and the production of short-chain fatty acids, but the microbiome plays an important role in the upper gut as well—for example, with lipid bioavailability. Investigators have used both mouse and rat models to study bile acid and other host-microbiome metabolic interactions related to lipid absorption. For example, Martin and colleagues (2007) measured bile acid signaling after transferring human baby microbiomes into gnotobiotic (germ-free) mice and reported an increased emulsification potential and greater lipid bioavailability in the humanized mice compared to the normal mice. Studies with conventional versus germ-free rats have yielded similar findings (Swann et al., 2011).

Also using the mouse model, investigators have demonstrated that introducing probiotics, such as *Lactobacillus paracasei* or *L. rhamnosus*, can induce differential metabolic responses (Martin et al., 2008a). Introducing

prebiotics in combination with probiotics also induces differential metabolic responses (Martin et al., 2008b).

Another body of evidence indicating that the microbiome plays a key role in human metabolism comes from research on bariatric surgery in both animal models and humans. Roux-en-Y gastric bypass (RYGB), the gold standard for bariatric surgery, has been associated with an 80 percent reduction in diabetes within 24 hours of surgery. The procedure has also been associated with reduced risks of colonic and other cancers. Nicholson explained that because the diabetes is cured immediately (i.e., not after subsequent weight loss), there must be a biochemical explanation. Part of that explanation likely lies in the microbiome. Zhang et al. (2009) reported a massive increase in *Gammaproteobacteria* in RYGB patients, compared to normal and obese individuals. Using a rat model, Li et al. (2011b) also reported an increase in Gammaproteobacteria as well as a massive change in bile acid metabolism following RYGB. Other microbiome changes have been detected in RYGB rats as well. Nicholson observed that while the microbiome changes may not be "the key" to understanding the connection between bariatric surgery and changes in diabetes, cancer, or other disease risks, "they are certainly part of the gear box" (Holmes et al., 2011). One possible mechanism is the cytotoxic environment created in the gut following bariatric surgery, as evidenced by fecal extract toxicity (Li et al., 2011a).

Gut Microbial Activities Affect Drug Processing in the Host

One of the goals of a systems-level understanding of human biocomplexity is to realize the vision of personalized or, as Nicholson called it, "precision" health care (Mirnezami et al., 2012). Pharmacometabonomics is one component of that care, in Nicholson's opinion. He defined pharmacometabonomics as "the prediction of the quantitative outcome or effect of a biomedical intervention based on a pretreatment metabolic model." The approach is predicated on the concept of "metabolic hyperspace," where the position of an individual is dependent on a multitude of factors (genes, diet, microbiome) (Nicholson and Wilson, 2003). The closer two individuals are in metabolic hyperspace, the more physiologically similar they are and the more likely they are to behave in the same way when presented with a challenge (e.g., a drug or other therapeutic intervention). As an example of a potential pharmacometabonomic application, Clayton et al. (2006) demonstrated that drug toxicity could be predicted based on pre-intervention metabolic profiles of urine. Other research groups have reported similar findings (Phapale et al., 2010).

In addition to drug toxicity, pre-intervention metabolic profiling has also been used to predict drug metabolism. For example, Clayton et al. (2009) demonstrated an association between gut microbial metabolites and

acetaminophen (Tylenol) metabolism, with microbial excretion patterns partly determining whether an individual is a weak or strong "sulfater." Sulfation is one of two main pathways of acetaminophen metabolism, with weak sulfaters being poor metabolizers. Nicholson explained that *Clostridium* and other microbes produce 4-cresol, a structural analog to acetaminophen that saturates the sulfation system, making for a weak sulfater. Moreover, 4-cresol does not compete for sulfation only with acetaminophen, but with all hydroxylated drugs. Nicholson said, "It affects hundreds of different compounds.… One gut microbial enzyme actually has amazing effects on the metabolism disposition and potentially toxicity in a very large number of drugs."

Interestingly, Nicholson noted, autistic children cannot sulfate acetaminophen (Alberti et al., 1999). In fact, according to Nicholson, the ability to sulfate acetaminophen is one of the most statistically significant tests for autism. Again, there is evidence of a microbial connection. Finegood et al. (2002) demonstrated abnormal *Clostridium* in children with autism. Altieri et al. (2011) showed that children with autism have much higher levels of microbially produced cresol than normal children. Yap et al. (2010a) reported that even non-autistic siblings of children with autism have higher levels of cresol than non-autistic siblings of children without autism.

The competition between 4-cresol and acetaminophen is just one of many types of microbiome-drug interactions. Other interactions include primary metabolism of orally administered drugs (as the first genomes that oral drugs interact with are microbial, not human, genomes), induction of enzymes that metabolize drugs, and changes in bioavailability (e.g., by changing local pH and the ionization state of drugs).

"There is now an enormous amount of interest in the pharmaceutical industry in the modulated microbiome for changing the way that drugs work," Nicholson remarked. In fact, there is a great deal of interest in drug-targeting the microbiome itself (Jia et al., 2008). Wallace et al. (2010) showed that drugging the microbiome can alleviate the toxicity associated with the common colon cancer drug CPT-11.

Population Metabolic Phenotyping and Disease Risk

In addition to its potential role in personalized medicine, metabolic phenotyping has potential applicability at the population level. Holmes et al. (2008b) introduced the concept of metabolome-wide association studies (MWAS), the metabolic equivalent of GWAS, and showed significant geographic variation in metabolic phenotypes. The same variation has also been associated with varying risks of cardiovascular and other diseases (Yap et al., 2010b).

OPEN DISCUSSION

The discussion during the question-and-answer period focused on the relevance of using animal models to understand the human microbiome, the relative importance of understanding microbiome composition versus function, and the dietary implications of individual microbiome variation.

Animal Models and the Human Microbiome

Of the mouse model, Nicholson said during his presentation, "This is the first time we have actually had a tool which allows us to measure quantitatively the response of complex organisms to things like probiotic or prebiotic interventions." During the question-and-answer period, several workshop participants asked questions or commented on the relevance of animal models to understanding the human microbiome. For example, there was a question about the implications of studying the impact of bariatric surgery on bile salt metabolism in rats, given that rats do not have a gall bladder and that human patients that undergo bariatric surgery have an increased incidence of gall bladder disease. Nicholson responded, "The important thing about these rat and mouse models is they help you to develop the tools for studying these complex interactions. We start to get a framework of what sort of pathways are interacting with what sort of bugs." Nicholson and colleagues are currently finishing a study of 100 bariatric patients, the results of which may indicate whether the rat model is predictive of humans.

Another audience member asked whether there might be a better animal model than mice or rats for studying the human microbiome. Lita Proctor noted that although the HMP was not able to use animal models as a complement to any of the human studies as per NIH Common Fund rules, the door is now open to the development of new animal models. However, it is not clear whether and how the HMP will move in that direction. Meanwhile, the National Institute of General Medical Sciences has been very interested in developing animal models, including some nontraditional animal models (e.g., zebra fish), for microbiome research.

There was also some discussion about the pig model being used to study the human microbiome. Nicholson observed that because of the similarities between pig and human physiology, developing the pig model might be an "important direction" for future research. Regardless of the chosen animal model and regardless of whether the goal is to simulate human disease or a "normal" human microbiome, he emphasized that the metabolic profile generated should approximate a human metabolic profile.

Microbiome Composition Versus Function

Questions and comments about the relative importance of "Who is there?" and "What are they doing?" were interwoven throughout the discussion. One participant suggested that instead of a "core" microbiome, perhaps there are "core" biological functions of the microbiome. She wondered whether any dietary or other functional microbiomic phenotypes have evolved over time. Another audience member questioned whether it is necessary to change the composition of the microbiome flora in order to induce a biological change, or whether changing the metabolites is sufficient. Nicholson replied, "I see no reason why you should have to do that. What you want to do is change the functional capacity of the microbiome, which means changing the interactions." He noted that the probiotic and prebiotic studies that he had mentioned during his presentation (Martin et al., 2008a,b) demonstrated significant metabolic changes in the microbiome despite the fact that actual microbial populations were altered only a "little bit." The same is true of yogurt, he said. When individuals consume yogurt, the few billions of microbes in that yogurt do not change the composition of the few trillions of microbes in your microbiome. Yet, they induce huge metabolic changes. He said, "I think data already exist that probiotics and prebiotics change the existing microbiome function."

Proctor agreed that inducing a biological change does not require a change in microbiome composition. However, recent evidence suggests that only about half of the gut microbiome is actually active at any given time, so it might be helpful to know which part of the microbiome is active. Nicholson added that another factor to consider is potency. Not only are some microbes active while others are not, but also some are more active than others. He said, "Like in any ecology, there are some very common species that don't do much, and there are few rare species that actually are linchpins in the ecology."

Dietary Implications of Individual-Level Microbiome Variation

An audience member asked about the dietary implications of individual variation in microbiome composition and function. As scientists learn more about the microbiome, how it varies among individuals, and how that variation impacts health and disease, will it become necessary to redefine or reestablish nutrient requirements? How long will it take before microbiome variation will have to be considered in a regulatory forum? Proctor agreed that the field could move in that direction. She mentioned recent evidence indicating that a gut microbe is able to produce riboflavin (vitamin B_2), raising questions about how much riboflavin our microbiomes supply, as opposed to how much we gain through diet, and what substrates

or nutrients stimulate that production. Nicholson added that not only do gut microbes produce vitamins, but they also compete for iron, other nutrients, and calories, especially in infants. He encouraged more research on the interaction between the infant microbiome and diet. "Whatever you put into the diet for the babies," he said, "the bugs might change what happens to that in quite significant ways."

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Interaction Between the Microbiome and Health and Environment

Two major overarching themes that emerged early during the course of the workshop were the intimate role the microbiome plays at the interface between humans and their environment and the key role the microbiome plays in human health. This chapter summarizes the presentations that explored in detail the environment-human-microbiome dynamic in the early years of life (i.e., how vaginal versus cesarean [C-section] deliveries impact the fetal microbiome and are associated with fetal health), in the oral environment (i.e., how periopathogens cause oral disease by interfering with the community in a subversive way), and in the adult gastrointestinal (GI) tract (i.e., how the indigenous microbiome influences the effect of a pathogen).

OVERVIEW OF PEDIATRIC CLINICAL IMPLICATIONS AND INTERVENTIONS[1]

One of the first studies conducted on early development of the human intestinal microbiome revealed some interesting findings, including that a baby's first stool contains microbes and that a baby's first antibiotic treatment has a marked effect on microbes in the GI tract (Palmer et al., 2007). More recently, Koenig et al. (2011) demonstrated in a case study of one infant how microbial diversity in the GI tract increases over time during the first year of life, with the introduction of specific types of foods causing phylum-level changes in microbial composition. Koenig et al. (2011) also

[1] This section summarizes the presentation of Josef Neu.

reported having found microbial DNA in the meconium (i.e., first stool). Josef Neu speculated on the implications of these and other recent microbiome research in pediatric populations. For example, that the first stool contains microbes suggests not only that a fetal microbiome exists, but also that its existence could relate to prematurity.

Fetal Microbiome: Clinical Implications

Evidence of microbes in the meconium refutes the popular notion that the mammalian fetal intestine is sterile and that the first exposure to maternal microbiota occurs during passage through the birth canal, according to Neu. Goldenberg and colleagues (2000) suggested that first exposure could occur during the last trimester of pregnancy, that is, babies are bathed in amniotic fluid that may contain microbes that have ascended from the vagina and translocated through the choriodecidual membrane. DiGiulio and colleagues (2008) examined the possibility by analyzing stored amniotic fluids of babies born at various gestational times. Using both culture-based and polymerase chain reaction (PCR) techniques, they found that gestational age was inversely correlated with microbial presence and quantity. In other words, babies born prematurely had more microbes in their amniotic fluids. The researchers also reported a positive correlation between both their culture and PCR results and amniotic fluid concentrations of white blood cells and interleukin-6 (IL-6), suggesting that microbial presence and quantity are associated with intestinal inflammation. Nanthakumar and colleagues (2000) had previously reported an inverse relationship between maturity and IL-8 expression, also suggesting that prematurity could be associated with an intestine-derived inflammatory response to microbes swallowed by the fetus through the amniotic fluid.

Together, these data suggest that when microbes are swallowed by the fetus, the ensuing infection increases the output of inflammatory mediators (e.g., IL-6, IL-8) and thereby potentially triggers premature labor as well as other problems (e.g., necrotizing enterocolitis, chronic lung disease, neurodevelopmental delays). Because amniotic fluid is difficult to sample, the next best evidence available for testing this hypothetical scenario comes from the baby's first stool. "If you think of it from the perspective that the baby's meconium is actually a reflection of what has been going on in utero in terms of the swallowing of the microbes," Neu said, "meconium could potentially be a very valuable source of information." Using data from meconium samples, he and his colleagues reported lower microbial diversity among less mature babies (Mshvildadze et al., 2010). More recent, unpublished data also show correlations between gestational age and phylum-level diversity—for example, a fairly strong negative correlation between gestational age and Actinobacteria (Triplett and Neu, unpublished

data). Neu noted that the *Gardnerella* genus (which is in the Actinobacteria phylum), when it is associated with bacterial vaginosis, has been associated with premature delivery.

Diseases in Preterm Babies: Role of the Microbiome

About 7 percent of babies in neonatal intensive care units (ICUs) who weigh less than 1,500 grams develop necrotizing enterocolitis (NEC). About 30 percent of babies who develop NEC do not survive. Of those who do survive, about 50 percent suffer significant neurodevelopmental delays. Symptoms include abdominal distention, redness around the belly button, and specific X-ray findings (Neu and Walker, 2011). Surgical treatment for NEC often results in a shortened gut, which requires about $1.5 million in medical care during the first 5 years of life.

In an ongoing microbiota study of babies with NEC, Neu and colleagues have been collecting weekly stool samples from NEC babies and carefully matched non-NEC babies (i.e., matched with respect to gestational age, size, time in the neonatal ICU). Their first results revealed differences in demography (i.e., babies with NEC were more likely to be formula-fed than breast milk–fed), antibiotic administration (i.e., babies with NEC were administered more antibiotics than control babies were), and fetal microbiota (Mai et al., 2011). With respect to fetal microbiota, the NEC and control babies demonstrated a marked difference in Firmicutes prevalence one week before diagnosis (60.68 percent in NEC babies, compared to 31.49 percent in controls) and a marked difference in Proteobacteria composition within 72 hours of diagnosis (70.9 percent in NEC babies, compared to 31.49 percent in controls). At the species, or operational taxonomic unit (OTU), level, there appear to be significant differences in *Klebsiella* spp. and *Cronobacter* spp.

Another recent study reported similar findings with respect to the relationship between antibiotic administration and NEC, with greater antibiotic use increasing the risk of NEC (Alexander et al., 2011). According to Clark and colleagues (2006), antibiotics are among the top 10 drugs administered to babies in neonatal ICUs, with about 95 percent of all babies being administered at least 48-72 hours of either ampicillin or gentamicin. As reviewed by Preidis and Versalovic (2009), an association between antibiotic administration and lower microbial species diversity has been observed in infants.

Late-onset sepsis is another prevalent disease among premature babies, affecting about 37 percent of babies born at less than 28 weeks' gestation, with fetal microbiota associations. According to Neu, coagulase-negative *Staphylococcus* spp., *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp., and *Enterococcus* spp. are the most common microorganisms in blood

cultures of babies with late-onset sepsis. Unpublished data from Neu's research group show lower overall microbial diversity in sepsis babies 2 weeks before diagnosis, with few Proteobacteria detected. At the onset of sepsis, Proteobacteria bloom, which Neu observed is similar to what happens with NEC.

C-Section Versus Vaginal Delivery: Microbiome Differences

Neu explored in more detail a subject that Lita Proctor touched on, that is, microbiome differences between babies born vaginally versus those born via C-section. It is an important topic, Neu argued, because of the impact of the microbiome on development of the immune system during the first year of life and because of the growing number of C-section deliveries worldwide. In the United States, C-sections have increased from 24 to 34 percent over the past 15 years; in large cities in China, C-section delivery rates reach 60 percent; and in some South American countries, for example, Argentina and Brazil, C-section deliveries in private hospitals approximate 100 percent. Neu and Rushing (2011) listed a range of health outcomes associated with C-section deliveries, including allergic rhinitis, asthma, celiac disease, diabetes mellitus, and gastroenteritis.

With C-section delivery, lack of exposure to the vaginal microbiota results in "abnormal" microbial seeding of the GI tract and "abnormal" development of immunity, according to Neu. Dominguez-Bello and colleagues (2010) reported that with vaginal delivery, the baby's first stool microbiota closely resembled the mother's vaginal microbiota, whereas with C-section delivery, the baby's first stool microbiota closely resembles the mother's skin microbiota. Neu mentioned some unpublished data that show not only differences in microbial presence between C-section and vaginal deliveries, but also changes in those differences over time. Major phylum-level differences that exist at week 1 (e.g., greater *Proteobacteria* abundance in C-section babies, greater Bacteroides abundance in vaginal babies) disappear by week 4, while certain genus-level differences that are not present at week 1 emerge at week 4 (e.g., relative abundance of *Enterococcus*).

Pediatric Microbiome: Implications for Long-Term Health and Disease

In conclusion, Neu briefly described yet another early-life disease, type 1 diabetes, that has been associated with the pediatric microbiome. In addition to genetic predisposition and other factors, researchers have found significant differences in microbial ecology between children who develop type 1 diabetes and children who do not (Brown et al., 2011; Vaarala et al., 2008). Butyrate-producing bacteria appear to be especially important for maintaining a healthy gut and preventing type 1 diabetes (see Figure 3-1).

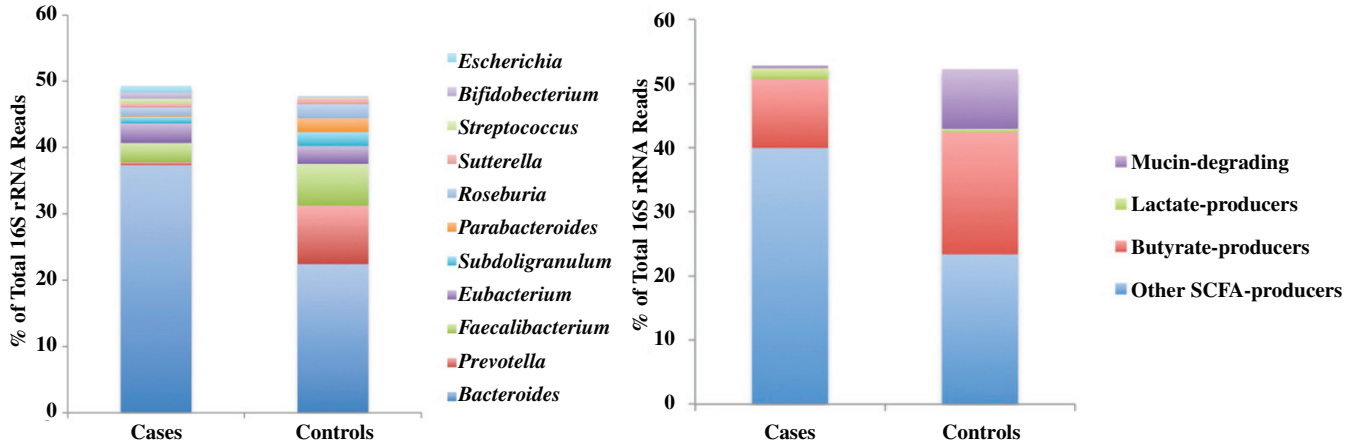

FIGURE 3-1 Differences in genus-level microbiome content between children who develop type 1 diabetes and children who do not.
NOTE: rRNA = ribosomal RNA.
SOURCE: Brown et al., 2011.

IMPACT OF MICROBIOME ON ORAL HEALTH AND DISEASE[2]

The oral environment operates under a "different paradigm" from other parts of the GI tract, according to Richard Darveau. A key difference between the oral and intestinal environments, one with significant implications for differentiating health and disease, is that two-way communication between the inside and outside environments is a regular feature of even a healthy oral cavity. Unlike the intestinal epithelium, which is characterized by tight junctions, junctional epithelium in the oral cavity is very loosely organized. The looseness allows for constant neutrophil movement from the vasculature to the gingival crevice. Elsewhere in the GI tract, neutrophil movement is a sign of inflammation or disease. In the oral cavity, it is "normal." Similarly, inflammatory cytokines are widely present in healthy mouths, where they play a key role in healthy tissue development and function. There are just "a lot more of them" in diseased mouths, Darveau explained. So the innate immune defense system is highly active even in healthy tissue. For example, Yoshioka and colleagues (2008) showed that plaque from both clinically healthy and diseased sites can stimulate both Toll-like receptor-2 (TLR-2)-mediated and TLR-4-mediated inflammatory responses. Darveau described disease as a "disruption in homeostasis," that is, a disruption in the healthy relationship between oral microbes and the host tissue—one that causes increased inflammation and, eventually, bone and teeth loss (Darveau, 2010).

Constant movement across the junctional epithelium in the oral cavity, combined with the fact that the periodontium is a highly vascularized tissue, implicates periodontitis as a contributing factor to systemic disease. Darveau remarked that while the mechanisms are still unclear, researchers have reported clinical associations between dental and systemic diseases (Zelkha et al., 2010).

Another important difference between the oral and gut microbiomes is the ease of sampling the former. Scientists have conducted "thousands and thousands of analyses" of the oral microbiome, according to Darveau, providing the data to paint a good picture of healthy versus diseased oral bacterial consortia. A healthy oral bacterial consortium is characterized by mostly Gram-positive bacteria, whereas a periopathogenic bacterial consortium is characterized by mostly Gram-negative bacteria. Years ago, Darveau was involved in work that led to the identification of three of these Gram-negative bacteria collectively known as the "red complex bacteria": *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. The three species are associated both with each other and with periodontitis (Socransky et al., 1998). Much of Darveau's research is on *P. gingivalis*, a

[2] This section summarizes the presentation of Richard Darveau.

late colonizer in ecological succession of the oral microbial biofilm that lands on the top layer of already formed biofilms (Zijnge et al., 2010).

Health as a Homeostatic Relationship Between Commensal Bacteria and Their Host

Initially, Darveau hypothesized that *P. gingivalis* produces a potent inflammation-inducing lipopolysaccharide (LPS). However, as described in Al-Qutub et al. (2006), he and his research team found that *P. gingivalis* produces a very complex LPS, one with a lot of structural heterogeneity and specific structural alterations that actually result in reduced inflammation under certain conditions (e.g., hemin concentration can influence LPS structure). So contrary to initial suspicion, Darveau said that *P. gingivalis* LPS "is very weak at activating inflammation." In some cases, it actually inhibits TLR-4-mediated inflammation (Coats et al., 2005, 2007). Other researchers have confirmed Darveau's findings, showing in a similar fashion that *P. gingivalis* is not only not a strong inducer of inflammation, but also an excellent modulator of host inflammatory response (Darveau, 2010; Hajishengallis et al., 2011).

Refutation of his initial hypothesis led Darveau to propose a new hypothesis: that *P. gingivalis* is a keystone species in the oral microbiota and that it impacts the host immune system not directly, but by subverting innate immunity in a way that prevents the host from detecting and clearing not just *P. gingivalis* but other oral microbes as well (Darveau, 2009, 2010). For example, by inhibiting TLR-4-mediated inflammation, *P. gingivalis* might be inhibiting the ability of TLR-4 to sense not only its own presence (and clear *P. gingivalis*) but also the presence of other microbes. Darveau noted that *P. gingivalis* can disrupt host tissue homeostasis via other mechanisms as well, for example, by inhibiting host cell secretion of the chemokine IL-8 in response to other oral microbes, not just in response to *P. gingivalis* (Darveau et al., 1998). Keystone species do not need to be present in large numbers, Darveau said, in order to exert global effects on the community.

To test the concept of *P. gingivalis* as a keystone species, Darveau and colleagues colonized both wild-type and germ-free mice with *P. gingivalis* and detected alveolar bone loss and an increase in total oral bacterial load in the wild-type but not the germ-free mice after 6 weeks (Hajishengallis et al., 2011). The results suggest that commensals must be present in order to induce a diseased state. Wondering if commensals alone could cause bone loss, Hajishengallis et al. (2011) also co-caged germ-free with wild-type mice (i.e., uninfected with *P. gingivalis*) and measured bone loss after 16 weeks. They reported that, yes, the germ-free mice showed bone loss after 16 weeks of having been co-caged with wild-type mice. Thus, naturally

existing commensals can cause eventual bone loss even in the absence of infection with *P. gingivalis*, but *P. gingivalis* does accelerate bone loss (i.e., when *P. gingivalis* is present, bone loss occurs at 6 weeks versus 16 weeks). Additionally, Hajishengallis et al. (2011) showed that both *P. gingivalis*-induced and natural bone loss require complement.[3] Complement receptor knockout mice showed no signs of bone loss even in the presence of *P. gingivalis*. Together, these results suggest that *P. gingivalis* accelerates natural bone loss by exploiting and modulating naturally existing commensal interaction with complement.

Recent, unpublished data in mice underscore the important role that commensals play in periodontal disease (Zenobia et al., manuscript in preparation). The data indicate that CXCL1 (a mouse analog of human IL-8) is expressed in both conventionally reared and germ-free mice, but that expression of CXCL2 (another mouse analogue of human IL-8) requires the presence of commensals. In mice, both CXCL1 and CXCL2 are needed for the "normal" neutrophil migration that characterizes a healthy oral environment. In humans, IL-8 is believed to be a key mediator in tissue production. Darveau concluded that we "need to know more concerning oral commensal bacteria contribution to health." For example, which oral commensal bacteria contribute to neutrophil migration in health? Can modulation of commensal bacteria improve health in certain individuals?

IMPACT OF MICROBIOME ON GASTROINTESTINAL HEALTH[4]

Medical students today are learning how to think about microbes in a different way from when Vincent Young was a student. "Find the bug, find the drug, because the only good bug is a dead bug" was the mantra, a way of thinking that originated with Koch's postulates (1882).[5] However, microbes play a much more complex role in human health and disease than previously thought. Today, Young is teaching his students to think not about "bad" and "good" bugs, but rather good and bad *communities* of microbial organisms.

[3] The complement system comprises about 25 proteins that work together to assist, or complement, the action of antibodies in destroying bacteria.

[4] This section summarizes the presentation of Vincent Young.

[5] Young's rendition of Koch's postulates was that the pathogen must be found in all cases of disease, the pathogen must be isolated from the host and grown in culture, the pathogen must re-create disease when given to a susceptible host, and the pathogen must be re-isolated from the experimental host.

A New Way of Thinking About Microbes: *Clostridium difficile* as a Case Study

Young presented a "case study" of *Clostridium difficile* infection to illustrate this change in paradigm. *Clostridium difficile* was associated with disease in the 1970s, when researchers fulfilled Koch's postulates to identify *C. difficile* as the causative agent of clindamycin-associated colitis (Bartlett et al., 1977). The case involved a 56-year-old man with chronic obstructive pulmonary disease (COPD) due to long-term cigarette use. The man was admitted with probable pneumonia and, as is standard of care for patients with suspected pneumonia, he was treated with broad-spectrum antibiotics. Although his pulmonary disease improved with antibiotics, on hospital day 3, the patient developed abdominal pain, diarrhea, and hypotension and was transferred to the intensive care unit, all as a result of a *C. difficile* infection. This is a "typical case," of *C. difficile* infection, Young said, where antibiotic treatment for one infection results in infection with the intestinal pathogen.

The "dogma" regarding *C. difficile* that Young was taught as a medical student was that the indigenous microbiota somehow prevents colonization by *C. difficile*. Accordingly, *C. difficile* erupts when antibiotics disturb the indigenous microbiota; colonization resistance against *C. difficile* is lost; and the patient is susceptible to spores of the pathogen, which are present in the hospital environment. When patients start showing signs of *C. difficile* infection, they are typically prescribed yet another antibiotic, usually metronidazole or vancomycin. Although this antibiotic treatment directed against *C. difficile* generally results in improvement, there can be problems with recurrence, with about 25 percent of patients redeveloping symptoms after ending antibiotic treatment. Importantly, recurrence can develop even in the absence of any further original antibiotic treatment and is thought to reflect continued imbalance in the microbiota that does not correct after stopping antibiotics. Although these hypotheses regarding the relationship between *C. difficile* and the indigenous microbiota were proposed shortly after it was proven that the pathogen caused antibiotic-associated colitis, they have only recently been examined experimentally.

Young challenges his students to consider other ways to think about *C. difficile*, reminding them that the indigenous gut microbiome not only has massive metabolic capacity, but also serves many vital functions. Importantly, it has been proposed that one of those functions is a protective one and that indigenous microbiota confer on the gut what Young called "colonization resistance." Without any additional insult to the microbiota, an estimated 25 percent of treated *C. difficile* patients do not have enough colonization resistance to withstand continued exposure to *C. difficile*

(Maroo and Lamont, 2006). "So there is something wrong," Young said. The question is, What?

Young and his research team have collected data demonstrating that individuals who have recurrent disease have lower diversity of their indigenous gut microbiota compared to individuals who do not and to healthy individuals. Specifically, Chang and colleagues (2008) examined the diversity of the gut microbiota using culture-independent methods that involve retrieving 16S ribosomal RNA (rRNA)-encoding gene data to distinguish different bacteria. The analysis of these data was accomplished by constructing what are known as rarefaction curves[6] for gut microbiota in three groups of patients: (1) individuals successfully treated for *C. difficile* with a single round of metronidazole or vancomycin, (2) individuals with recurrent disease, and (3) controls. The rarefaction curves showed that individuals with recurrent disease had the least amount of gut microbial diversity. Although the gut microbiome diversity in individuals who were successfully treated for *C. difficile* was not markedly different from that of the controls, it was at the lower end of what would be considered "normal." However, intestinal microbial diversity in patients with recurrence was much lower than in the other two groups.

This new knowledge that refractory *C. difficile* disease is associated with lower gut microbiome diversity helps explain the efficacy of an "alternative" treatment for *C. difficile*, which has been known of for years but has had a recent resurgence given the increasing burden of *C. difficile* infection. Instead of administering repeated courses of antibiotics in an attempt to kill the "bad" bug that keeps reappearing, physicians try to treat recurrent *C. difficile* with what is known as microbiota transplantation. By administering a new microbiota (in the form of the administration of fecal material from a healthy individual), the intention is to restore microbiota diversity and therefore colonization resistance. Despite the obvious "ick factor" of this treatment, it has become an option for patients with multiple recurrences with a greater than 95 percent success rate, according to Young (Gough et al., 2011).

[6] Young explained that rarefaction analysis is a tool from classical ecology that provides a general sense of the abundance of different species or, in the case of 16S microbiome data, operational taxonomic units (i.e., bacterial types defined by similar 16S-encoding gene sequences). Rarefaction curves are created by repeatedly sampling the data and plotting the number of unique observations as a function of sampling effort. As the number of samples increases, the number of unique observations decreases. An exhaustive sampling of a community yields a flat curve, indicating that no new species should be identified no matter how many additional samples are taken. When comparing two rarefaction curves derived by sampling two communities, the curve that lies below the other at a given level of sampling indicates that the community from which the curve was derived is less diverse.

Resilience of Gut Microbial Community Structure

How does colonization resistance become compromised in the first place? Evidence suggests that insults to the microbiome, such as antibiotic treatment, can have long-lasting effects on the indigenous microbial community. Young and colleagues treated mice with antibiotics, let the animals recover from the antibiotic stress in a sterile environment, and then observed what happened when they were either left alone in a sterile environment or co-housed with a donor mouse (Antonopoulos et al., 2009). They found that mice left alone, with no donor mouse present to repopulate their guts, had microbiota that looked very similar to each other but very different from microbiota in mice that had been co-housed with donors. Even 6 weeks after stopping antibiotic treatment, mice left alone had much lower microbiota diversity than the other mice. However, as with humans, if fecal transplantation is done, diversity can return to normal.

Subsequent mouse research showed that with respect to *C. difficile* infection, colonization resistance can be overcome by the administration of specific (but not all) antibiotics or combinations of antibiotics. Although early work with antibiotic-treated mice was unsuccessful in modeling human *C. difficile* infection, Chen and colleagues (2008) were able to establish disease by pretreating the mice with a cocktail of five antibiotics before treating them with clindamycin and challenging them with *C. difficile*. Young's team recreated the Chen et al. (2008) model and found that a pretreatment of five antibiotics without clindamycin did not cause disease, that clindamycin alone without pretreatment allowed transient colonization without disease (i.e., the infected mice shed bacteria briefly but showed no signs of inflammation), and that the combination of the pretreatment antibiotic cocktail followed by clindamycin allowed *C. difficile* colonization and the development of disease (Reeves et al., 2011). The severity of disease in the cocktail-plus-clindamycin treatment group varied. About half of the animals became very ill clinically, while the other half were able to maintain their health even though their gut epithelia became inflamed. The sicker animals also had more *C. difficile* and bacterial toxin present in their intestinal tissue.

With respect to microbial taxonomic composition, researchers observed high levels of the Firmicutes families, especially Lachnospiraceae genera (i.e., important short-chain fatty acid producers), and some Bacteroidetes families in untreated mice, but mice in the cocktail-plus-clindamycin treatment group bloomed Proteobacteria (e.g., *E. coli*, which Young described as only a "minority player" in a healthy gut). The microbiota in mice that developed clinical illness remained dominated by Proteobacteria over time, while the microbiota of mice that suffered some inflammation but did not become clinically ill eventually reverted to "healthy" Lachnospiraceae-dominated communities (Reeves et al., 2011, 2012).

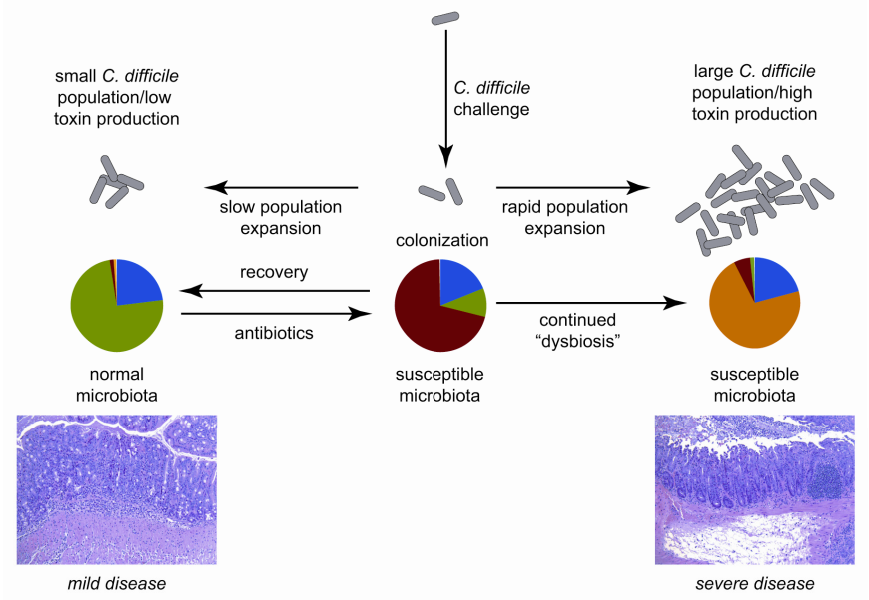

FIGURE 3-2 Model of the interaction between dynamics of the gut microbiota and *C. difficile* in antibiotic-treated mice, with clinical outcome being determined by the balance between recovery of the indigenous gut microbiota following antibiotic withdrawal and growth of the *C. difficile* population.
SOURCE: Reeves et al., 2011.

Young's team repeated the experiment using cefoperazone instead of clindamycin and observed that all mice administered cefoperazone died as soon as they were infected with *C. difficile*. Moreover, colonization resistance was lowered so much that their microbiomes became pure cultures of *C. difficile*, and the microbiota were unable to restore colonization resistance even after some recovery time.

Young's interpretation of the results is that colonization resistance recovery following an antibiotic assault seems to depend on which is happening faster—growth of the indigenous microbiota or growth of *C. difficile* (see Figure 3-2). Restoring balance in the community, or preventing imbalance, could be the basis for yet another new therapeutic approach to managing *C. difficile*. For example, Young mentioned the dissertation research of one of his students demonstrating that Lachnospiraceae bacteria are associated with greater colonization resistance. He wondered whether restoring balance might be simply a matter of adding "more bugs" in the "right combination."

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4

Influence of the Microbiome on the Metabolism of Diet and Dietary Components

Although research on the microbiome is considered an emerging science, scientists already have made tremendous progress in understanding the microbial makeup of the microbiome and in associating microbiome diversity with human disease. Moreover, they are beginning to make headway in understanding *how* the microbiome impacts human health and disease. It is likely that much of this impact is mediated through diet. Growing evidence suggests that gut microbes influence what the human host is able to extract from its diet, both nutritionally and energetically. This chapter summarizes the workshop presentations and discussion that were focused on the influence of the microbiome on diet and dietary components.

DIET, OBESITY, AND THE GUT MICROBIOME[1]

When the tremendous amount of undigested polysaccharides, lipids, and peptides that pass through the small intestine unabsorbed enter the large intestine, they serve as the perfect medium for growing a rich gut microbiota. "It stands to reason," Peter Turnbaugh said, "that diet is going to play an important role in shaping the ecology and function of this community." But how? And how does the gut microbiome, in turn, contribute to dietary energy harvest? Despite considerable interindividual variation in gut microbiome species, all individuals share a core set of microbial genes, according to Turnbaugh. What additional functions, or metabolic capa-

[1] This section summarizes the presentation of Peter Turnbaugh.

bilities, do these genes afford their human host? How do they allow for metabolism of all of the undigested polysaccharides and other substances inaccessible by human enzymes? And how does that impact human health and disease? Turnbaugh summarized results from a series of experiments designed to address these questions, with a focus on obesity.

Impact of Gut Microbiota on Energetics and Obesity

Turnbaugh's interest in obesity was sparked by work conducted in Jeffrey Gordon's laboratory at Washington University, where Fredrik Backhed and colleagues compared body fat in germ-free mice (i.e., mice raised in an isolator and without any exposure to microbes) to body fat in conventionally raised mice (i.e., mice that had been raised their entire life exposed to microbes) (Backhed et al., 2004). Researchers reported lower total body fat in the germ-free mice but were able to recover the body fat by colonizing germ-free mice with microbial communities harvested from conventionally raised mice. In Turnbaugh's opinion, the most interesting finding of the study was that conventionally raised mice had more body fat even though they were consuming fewer calories. This was true for both female and male mice and across multiple genetic backgrounds.

Turnbaugh was curious about this "perplexing" phenomenon. How does the microbiome affect the ability of its host to harvest energy from the diet? He and colleagues conducted some studies using 16S ribosomal RNA (rRNA) sequencing to identify phylum-level bacteria in the microbiomes in two different mouse models, *ob/ob* mice (i.e., mice that chronically overeat because they are genetically deficient in leptin) and diet-induced obese mice (i.e., genetically identical mice that are fed a diet high in fat and simple sugars) (Ley et al., 2005; Turnbaugh et al., 2006, 2008). With both models, the researchers found that lean mice had a moderately greater proportion of Firmicutes (60 percent) than Bacteroidetes (40 percent) but that obese mice had an even greater proportion of Firmicutes than Bacteroidetes. Thus, obesity correlates with increased Firmicutes and decreased Bacteroidetes.

What is the nature of the association? Are the altered microbial communities affecting their hosts in different ways? To answer this question, Turnbaugh and colleagues conducted a microbiota transplantation experiment, where they started with a panel of germ-free recipient mice, all of the same weight and age and with similar other features, and colonized the mice with microbiota samples taken from either an obese or a lean mouse donor (i.e., both *ob/ob* and diet-induced obese donors). Then they observed the impact of the transplantation over time (Turnbaugh et al., 2006, 2008). Researchers observed about twice as much gain in body fat in mice receiving microbiota transplanted from either *ob/ob* or diet-induced obese donors, compared to mice receiving microbiota from lean donors (see

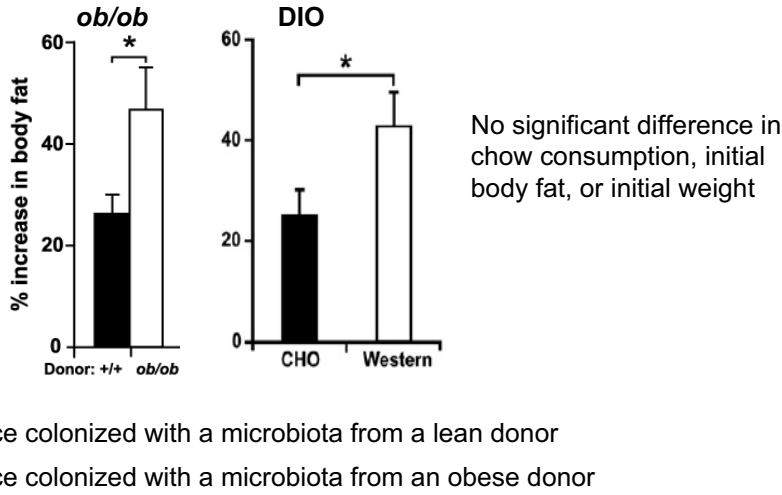

FIGURE 4-1 Difference in body fat gain between initially germ-free mice that receive a microbiota transplanted from either an obese donor (either a genetically obese donor [*ob/ob*] or a diet-induced obese donor ["Western"]) or a lean donor (again, either a genetically lean donor [+/+] or a diet-induced lean donor ["CHO"]).
NOTE: DIO = diet-induced obesity.
SOURCE: Turnbaugh et al., 2006, 2008.

Figure 4-1). Again, the mice that gained more fat tissue did so even though they were consuming the same amount of calories as the mice with microbiota from lean donors. These results suggest that microbial communities derived from obese versus lean mice impact the energy balance of their new hosts in different ways.

Human twin data provide additional evidence to support the hypothesis that the microbiome impacts host energetics. Using 16S rRNA data from both monozygotic and dyzygotic twin pairs, Turnbaugh and colleagues (2009a, 2010) found greater than 300 microbial genes associated with obesity. Many of these genes "make sense," Turnbaugh said, given the shift in the relative abundance of Firmicutes and Bacteroidetes associated with obesity in mice. Some of them also indicate a microbial contribution to host carbohydrate and other metabolic pathways.

In another human study, Greenblum et al. (2012) analyzed metagenomic data from twin pairs and other individuals and identified network-level differences in microbial metabolism genes between obese and lean individuals. That is, they identified specific networks of genes that were associated with obese individuals and other networks associated with lean individuals. Most of the differences between the obese- and lean-associated networks were on

the periphery of the networks. Turnbaugh explained that the periphery represents interaction with the host or environment (e.g., intake of a substance from the host or environment), as opposed to glycolysis or some other core component of metabolism. As was the case with the obesity-associated genes detected by Turnbaugh et al. (2009a, 2010), many of the obesity network genes are involved in carbohydrate metabolism as well as carbohydrate transport, nitrate reduction, and xenobiotic metabolism.

Impact of Gastric Bypass on Gut Microbiota: More Evidence of Co-Metabolism by Microbes and Their Host?

Evidence from gastric bypass surgery experiments suggests that gut microbiota impact more than host energetics—they impact host metabolism at large. Turnbaugh reiterated what Jeremy Nicholson had mentioned during his presentation about the metabolic consequences of gastric bypass surgery happening too quickly to be explained by the change in caloric intake. Turnbaugh was curious about the potential role of the gut microbiota as a mediator of these rapid metabolic changes. Using Roux-en-Y-operated mice and comparing them to two different types of controls (sham and weight-matched sham mice), Turnbaugh and colleagues (Alice Liou and Lee Kaplan, Massachusetts General Hospital) observed a significant difference in how quickly the microbial community structure changed after surgery. The microbiota of all the mice changed following their respective surgeries, but the microbiota of the Roux-en-Y-operated mice changed much more dramatically within the first week following surgery. Turnbaugh remarked that the next step is to see if some of the metabolic outcomes triggered by gastric bypass surgery can be transferred to germ-free mice via the gut microbiota.

Impact of Diet on the Human Gut Microbiota

Turnbaugh and colleagues (2009b) conducted an extensive set of diet shift comparisons using a "humanized" mouse model, that is, an initially germ-free mouse that was colonized with a human microbiome. They compared 16S rRNA sequences in humanized mice that were fed conventional mouse chow versus humanized mice fed a "Western" diet (i.e., high fat) and observed a rapid effect of diet shift on the gut microbiome, with only a single day of a high-fat diet having a significant effect. According to Turnbaugh, the results suggest that at least in the mouse model, the gut microbiome is "incredibly dynamic" and can respond to dietary perturbations very quickly. The researchers also found a significant effect of host diet on microbial gene abundance and expression.

The same data reported in Turnbaugh et al. (2009b) were further analyzed using a novel methodology described in Reshef et al. (2011) that enabled the researchers to identify and compare the strength of all possible relationships among all species-level operational taxonomic units (OTUs) across all samples. For example, one possible relationship is what the researchers called a "mutual exclusion" relationship, whereby a particular OTU is present in a microbiome only in the absence of another particular OTU. Reshef and colleagues (2011) concluded that a large proportion of the microbial species relationships could be explained by diet (i.e., conventional diet versus Western diet) as well as by sex and age. So there appear to be networks of diet-dependent microbial species.

Impact of Diet on Gut Microbiota in Other Mammals

The relationship between diet and the gut microbiota is not limited to humans and mice but extends across a wide range of mammalian species. Ley et al. (2008) reported differences in microbiome structure between omnivorous, herbivorous, and carnivorous species (among a total of 60 species, including humans). Subsequently, Muegge et al. (2011) collected data indicating that the microbial communities among these three different groups of mammals have evolved different suites of genes that allow their hosts to better process their respective diets. For example, gut microbiomes in carnivores tend to be enriched with amino acid catabolism genes.

Implications of the Association Between Diet and the Microbiome

Evidence collected by Turnbaugh and others suggests that the human ability to extract and store calories from food as fat is at least partially impacted by gut microbes. In turn, dietary choices impact the gut microbiome. This diet-microbiome interaction suggests to Turnbaugh that nutrition might be better viewed from a metagenomic perspective—one that takes into account both host and microbial genetics. It also raises questions about (1) the definition of a calorie (e.g., Do scientists need to redefine a calorie in relation to the gut microbiome?), (2) the future of personalized nutrition (e.g., Can nutritionists use knowledge of the human microbiome to design personalized diets?), and (3) next-generation medical treatments (e.g., Can medical researchers use this knowledge to design microbiome-targeted interventions?).

MICROBIAL METABOLITES OF DIETARY COMPONENTS[2]

When assessing diet-disease risk relationships, Johanna Lampe said, "We really can't ignore the contribution of the gut microbiome." Not only do gut microbes influence host energetics, as Peter Turnbaugh elaborated, they also play key roles in multiple other areas of host metabolism. Microbes contribute to host fermentation, reduction of nitrate and sulfate, esterification, aromatic fission, and hydrolysis and deconjugation (i.e., not just of glycosides in our plant food, but also of steroid hormones and other endogenous compounds that are excreted in bile and end up in the colon). Qin et al. (2010) identified a number of host metabolic pathways handled by gut microbes, many of which are involved with carbohydrate or amino acid metabolism or xenobiotic biodegradation. But how do microbes influence metabolites? And how do microbial metabolites of dietary components contribute to disease prevention and disease risk?

Lampe addressed these questions using xenobiotic degradation of phytochemicals as an example. There are an estimated 25,000 phytochemicals, with both negative and positive effects. As an example of a phytochemical with negative effects, most people associate nitrates with processed meats, but in fact vegetables are a major source of nitrates in the human diet. Water is another major source. Nitrates can be converted into nitrites, which in turn can interact with a number of different compounds to form nitrosamines, nitrosamides, and nitrosoguanidine; these, in turn, can form DNA adducts and cause DNA damage, creating the potential for carcinogenesis. On the flip side, there are a whole host of dietary bioactive phytochemicals with potential beneficial human health effects (Scalbert et al., 2011). These include the phenolics (phenolic acids, stilbenes, curcuminoids, chalcones, lignans, flavonoids, isoflavones), terpenoids (phenolic terpenes, carotenoids, saponins, phytosterols), organosulfurs (thiosulfinates), and nitrogen-containing compounds (glucosinolates, indoles). Lampe focused on three specific phytochemicals: glucosinolates in cruciferous vegetables, soy isoflavones, and plant lignins.

Glucosinolates and the Human Gut Microbiome

Cruciferous vegetables—that is, broccoli and its vegetable relatives—are the poster children of cancer-preventing vegetables. Both epidemiological and animal data show consistent associations between intake of cruciferous vegetables, whether broccoli, cauliflower, cabbage, or something else, and lower risks of various cancers, primarily of epithelial origin (e.g., lung, colorectal, breast, prostate, and pancreatic cancers). The epidemiological

[2] This section summarizes the presentation of Johanna Lampe.

data have shown associations across many population groups in Asia, Western Europe, and North America. Animal studies have shown that both cruciferous vegetable extracts and isothiocynate and indole isolates are chemopreventive. Various mechanisms have been proposed to explain the association, including decreased inflammation and oxidative stress, induced cell differentiation and apoptosis, and improved carcinogen metabolizing capacity.

From a human dietary perspective, one of the challenges to deriving chemopreventive benefit from cruciferous vegetables is that isothiocynate, the active component that actually imparts the protective benefit, is difficult to access. It exists in the plant as a glucosinolate, which the plant enzyme myrosinase cleaves into isothiocynate. However, myrosinase is active only in raw vegetables. Cooking inactivates it. On the basis of measurements of excreted total isothiocyanates in urine, Shapiro et al. (2001) reported that chewing uncooked broccoli results in much greater recovery of isothiocynates than swallowing unchewed sprouts and that cooking decreases isothiocyanate availability relative to both chewed and unchewed raw broccoli. They also found that pretreating cooked broccoli with myrosinase dramatically increases availability (i.e., to a point where excretion is more than double what it is with chewed uncooked broccoli). Lampe noted that one could thus argue the value of pretreating all cruciferous vegetables with myrosinase, but that would pose yet another challenge—that is, free isothiocyanate in the diet tends to cause gastritis, with nausea and vomiting, in some individuals. Also, it is not really clear where in the gastrointestinal (GI) tract, or how, isothiocyanates exert their chemopreventive effect and whether introducing pure isothiocyanate would be ideal.

Given that very few human populations regularly consume raw cruciferous vegetables, how are humans deriving the chemopreventive benefit of broccoli and other cruciferous vegetables? The answer, Lampe said, is in our gut microbiome. Lampe and her colleagues reported a wide range of recovery of isothiocynates (ITCs) in urine after eating 200 grams of cooked broccoli (Li et al., 2011). Some individuals excreted almost no ITC (i.e., the ITC was not available to them), whereas others excreted nearly 30 percent (i.e., indicating high availability). To determine whether the gut microbiome might be contributing to this variation, researchers analyzed fecal samples from the low- versus high-ITC excretors and observed that fecal bacteria in the low-ITC excretors have a lower capacity to degrade glucoraphanin compared to the high-ITC excretors. So clearly there is something going on at the level of the gut microbiome, Lampe remarked, with high excretors containing the enzymatic machinery necessary for cleaving glucosinolate into ITC. The researchers did not find any major taxonomic differences in bacterial composition. However Li et al. (2011) was a pilot study, Lampe

remarked. She hopes to examine bacterial composition differences between low- and high-ITC excretors in more detail in a future study.

Bacterial Metabolism of Daidzein, a Soy Isoflavone

Soy protein has generated long-standing interest for its potential effects on bone loss and hot flashes in perimenopausal women because of the weak estrogenic properties of the two major soy isoflavones, daidzein and genistein. Like many flavonoides, isoflavones are metabolized by gut bacteria.

Daidzein can be metabolized in two ways, via either the formation of equol, which is an isoflavone, or the formation of *O*-desmethylangolensin. Only about 30 to 50 percent of individuals produce equol, depending on gut microbial composition and depending on the population. For example, in Asia, the percentage of individuals who produce equol is closer to 50 percent, compared to the United States, where it is closer to 25 to 30 percent. Interestingly, Lampe noted, the percentage of individuals in Japan that produce equol appears to be decreasing and is now down to about 30 to 35 percent. It is unknown whether the shift is a result of dietary and associated gut microbiome changes in the younger generation. While not everyone produces equol, most individuals produce *O*-desmethylangolensin. To determine whether any specific microbial communities are associated with the capacity to produce equol, Hullar and Lampe (unpublished) identified individuals as equol producers or nonproducers based on a soy protein challenge, collected fecal samples, and analyzed 16S rRNA as part of what Lampe described as a "quick and dirty" evaluation of the gut microbiome. Their data suggest that fecal bacterial communities in equol producers differ from those of nonproducers. Moreover, within the equol producers, they found that equol production is associated with differences in the fecal microbiome makeup. Lampe speculated that several different bacteria consortia may be capable of equol production.

A number of research groups have looked at whether equol production is associated with any health outcomes. For example, Aktinson et al. (2003) and Frankenfeld et al. (2004b) reported positive associations between equol production and 2-OH/16alphaOHE1 (16alpha-hydroxyestrone) ratios in premenopausal and postmenopausal women. Frankenfed et al. (2004a) reported that mammographic density was 39 percent lower in equol producers. Akaza et al. (2002) reported that plasma equol concentrations were inversely associated with prostate cancer risk in Japanese men. Lastly, Fuhrman et al. (2008) reported a significant interaction between soy intake and equol-producer status in predicting breast density in postmenopausal women. Lampe noted that not all reported associations hold up across all populations. It is not clear why so many associations have been reported between equol production and disease risk in the Japanese population,

perhaps because of early life exposures and "priming" of the microbial systems.

Gut Microbial Metabolism of Plant Lignins

As a final example of the impact of the microbiome on host metabolism of dietary components, Lampe mentioned plant lignins. A whole host of plant lignins can be found in seeds, nuts, berries, grains, and other foods, most of which are metabolized into enterodiol and sometimes further converted into enterolactone. Kuijsten et al. (2005) reported highly variable enterodiol and enterolactone production among individuals. Lampe and colleagues measured microbiome diversity for low-, intermediate-, and high-enterolactone excretors among 115 women and detected significant differences between the low and high excretors and between the intermediate and low excretors. The greatest diversity was among high excretors and the least diversity among low excretors. suggesting that microbial diversity may be associated with enterolactone production. That diversity appears to be distributed across phyla, with high excretors having 20 unique genera.

BIOGEOGRAPHY OF THE GI TRACT

During the open panel discussion at the end of the first day, there was some discussion around the fact that most human microbiome studies to date are based on fecal sampling. Workshop participants expressed varying opinions about whether microbes and metabolites in the feces reflect what is happening in the gut. One audience member said, "I think you are looking in the wrong place, checking stool." He suggested sampling the small intestine, where bacterial overgrowth is a problem in patients with small-bowel disturbances. There are sampling technologies available, he said. Another audience member agreed and noted that he and his gastroenterology colleagues are beginning to collect these samples in some of their pediatric patients. Yet another audience member asked if anyone has ever compared feces microbiota to microbiota from various portions of the gut.

Vincent Young described feces as the "summary statement of your gut." In his opinion, feces has most of what exists elsewhere in the GI tract. It may not provide any indication of the relative abundance of species at various points upstream, but it does provide "some indication" of what is there. However, he emphasized that the usefulness of feces sampling depends on the research question. In some cases, it may not be a good choice. He noted that samples have been collected from various places along the length of the human GI tract and that they reveal both longitudinal and axial differences in both 16S rRNA and metagenomic microbial sequences.

Peter Turnbaugh remarked that based on his observations in mice, while

there are some cases in which a proximal gut sample can be distinguished from a distal gut sample, overall the picture derived from fecal samples is similar to that derived from colon or cecum sampling of either the mucosal or the luminal content. He noted data collected by Paul Eckburg and colleagues (2005) showing that microbial communities can be matched to individuals regardless of whether the microbial sequencing data came from biopsy or fecal samples. Turnbaugh speculated that some biogeographic structure probably exists, but at a finer scale.

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5

Influence of Diet and Dietary Components on the Microbiome

As the workshop progressed, speakers explored in greater depth the impact of diet on the microbiome, how dietary influences on the microbiome contribute to human health and disease, and ways to modulate the microbiome to build and maintain health through the use of prebiotics and probiotics in food products. This chapter summarizes that discussion.

HUMAN BREAST MILK[1]

> *Through evolutionary experimentation, mammals have spent the last 120 million years successfully developing "the most efficient, effective and adaptable means of postnatal nutrient provision that has ever arisen among vertebrates: lactation."* —Blackburn (1993)

That a majority of people suffer from diet-dependent diseases raises the question, Is it possible to prevent disease through diet? In his exploration of the preventive potential of the human diet, Bruce German focuses his research on the one food that evolved to be preventive: human breast milk. The cost–benefit trade-off associated with human milk is key to understanding milk's preventive potential, German explained. Everything in human milk costs the mother. "The mother is literally dissolving her tissues to make milk," he said. Yet the third most abundant component in milk, the oligosaccharides, are undigestible by the infant. How can this be? How

[1] This section summarizes the presentation of Bruce German.

can such an abundant material of such a costly phenotype be undigestible by the individual for whom it is intended, that is, the infant?

What Are Human Milk Oligosaccharides (HMOs), and What Health Benefit Do They Provide the Infant?

Glycobiology[2] is "disastrously, catastrophically complex," German said. According to German, the number of possible glycans, or oligosaccharides, in a biological system is in the billions, based on the number of ways that sugars (the basic structural units of glycans) and linkages (the bonds between sugars) can be combined. This makes sense given that oligosaccharides on cell surfaces are the basis of a recognition system across all life forms. Carlito Lebrilla developed a methodology for analyzing glycan complexity in human milk, based on innovative separation technologies and very high-efficiency, high-accuracy mass spectrometry (Ninonuevo et al., 2006). His research team has constructed an annotated database of the nearly 200 highly variable structural compositions of HMO (Wu et al., 2010, 2011).

David Mills was among the first to address the question, What do HMOs do? He hypothesized that HMOs serve as a food source for the infant microbiome. However when he and his colleagues tested bacterial growth on HMO as a sole food source, none but *Bifidobacterium infantis* grew (Ward et al., 2007). "Perhaps we shouldn't be surprised," German said, given that *B. infantis* is a dominant member of the breast-fed-infant microbiome. Moreover, Mills and his group have discovered that only very specific strains of *B. infantis* grow on HMO medium. Even bacteria that grow very well on a variety of other sugar media are unable to grow on HMO medium (Marcobal et al., 2010; Ward et al., 2006). (The only other genus that appears to be able to grow on HMO medium is *Bacteroides*. However, as both German and, later during the question-and-answer period, Mills explained, *B. infantis* readily outcompetes *Bacteroides* when the two are grown together.) "What we are discovering about this remarkable interaction between milk oligosaccharides and this particular bacterium is remarkable," German said. "Mothers are literally recruiting another life form to babysit their babies and using the oligosaccharides to direct the microbiome."

How does the system work? For example, if oligosaccharides are serving as a food source for *B. infantis*, which oligosaccharides are being consumed? Mills and his group have discovered that unlike other bifidobacteria, *B. infantis* selectively cleaves and eliminates sialic acid–containing

[2] Glycobiology is the study of the structure, biosynthesis, and biology of glycans, also called oligosaccharides (i.e., sugar chains).

oligosaccharides (LoCascio et al., 2007; Ward et al., 2007). Only an estimated 4 to 38 percent of HMOs are sialyated; a higher proportion are fucosylated (40 to 70 percent) (Ninonuevo et al., 2006). Moreover, Mills's team has also identified which *B. infantis* genes cleave what HMO linkages (Sela et al., 2011). Interestingly, in German's opinion, the expression of the bacterial enzyme that actually cleaves the sialyated oligosaccharides is regulated by the abundance of HMOs in the growth medium. There is other evidence indicating that human milk sugars interact with the microbiome in ways that increase the value of the microbiome to the infant. For example, German mentioned the research of Helen Raybould's group on *B. infantis* and its role in endocrine signaling in the infant intestine (Chichlowski et al., 2012).

The real question, in German's opinion, is whether the association between HMOs and *B. infantis* persists as a phenotype in "real life." That is, "does is really influence the bacteria in living babies?" Data on microbiome development through the first 12 weeks of an infant's life show that initially *Bifidobacterium* is not present in the microbiome (manuscript in preparation), but by week 12 it emerges as a dominant member of the microbiome. Evidence from fecal sampling indicates that HMOs are not being digested during the first weeks of life, presumably because there are no bifidobacteria to digest them, but they begin to disappear from the infant feces at the same time *Bifidobacterium* begins to dominate the microbiome (manuscript in preparation). Thus, the association between HMOs and *B. infantis* is a "true symbiotic relationship," German said. "It's as important to feed the bacteria in the baby as the baby."

German suggested that knowledge of human milk–microbiome symbiosis could be translated into practice in several ways. For example, he mentioned Mark Underwood's research on the effects of administering a combination of *B. infantis* and HMOs to premature infants (manuscript in preparation).

HOST-MICROBE INTERACTIONS IN THE PERINATAL PERIOD[3]

There are very few data on the development of the gut microbiota in healthy infants, let alone how diet impacts that microbiota. Yet, there is a plethora of clinical and epidemiological data suggesting that breast-feeding promotes mucosal immune development and protects against many diseases. These data, combined with the fact that human milk contains a variety of bioactive proteins, carbohydrates, and lipids not present in infant formula, raise questions about whether and how the infant gut microbiota differs between breast-fed and formula-fed infants. Sharon Donovan's long-term

[3] This section summarizes the presentation of Sharon Donovan.

research goal is to use noninvasive approaches to define how early nutrition shapes host-microbe interactions and influences intestinal development in breast-fed versus formula-fed infants. She hopes that the knowledge gained can be used to identify selective additives, such as bioactive proteins, prebiotics (including HMO), and probiotics that can be added to infant formula to provide some of the health benefits afforded by breast-feeding.

Differential Expression of Microbial Genes in Breast-Fed Versus Formula-Fed Infants

In what Donovan described as a "proof-of-concept" study, she and colleagues used a method developed by Robert Chapkin (Davidson et al., 1995) for isolating exfoliated epithelial cells from stool to identify genes differentially expressed in breast-fed versus formula-fed infants (Chapkin et al., 2010). Specifically, they analyzed stool samples collected at 3 months of age from vaginally delivered term infants who were medically certified as healthy and who were either exclusively breast-fed ($N = 12$) or formula-fed ($N = 10$) (Chapkin et al., 2010). The researchers gained institutional review board (IRB) approval to train the mothers themselves to collect the samples at home. The initial messenger RNA (mRNA) expression microarray analysis yielded 4,250 genes that were expressed in all infants. Of those, about 1,200 were significantly differentially expressed between breast-fed and formula-fed infants. Due to the small sample size and thus greater potential for false discovery, the scientists compared these 1,200 genes to a list of 546 that they had predicted could be differentially expressed based on their known roles in intestinal biology. This yielded 146 differentially expressed genes, to which researchers applied a linear discriminant analysis and coefficient of determination analyses developed by Edward Dougherty and colleagues (Dougherty et al., 2009; Kim et al., 2000) to identify the genes that best classified breast-fed versus formula-fed infants and those that were master regulators, respectively.

The strongest classifier was *EPAS1*, which encodes a protein involved in cellular response to hypoxia. Given that necrotizing enterocolitis (NEC) is associated with tissue hypoxia and that human milk has been shown to protect preterm infants from NEC, Donovan speculated that upregulation of *EPAS1* in breast-fed infants might be helping those babies' guts to tolerate hypoxic episodes.

Other genes that qualified as good classifiers are summarized in Table 5-1. The linear discriminant analysis methodology used allowed investigators to identify not just single genes that could be considered good classifiers of breast-fed versus formula-fed infants, but also two- and three-gene combinations (Chapkin et al., 2010).

Donovan speculated that these gene expression differences might ex-

TABLE 5-1 Exfoliated Epithelial Cell Genes Identified as Good Classifiers of Breast-Fed (BF) Versus Formula-Fed (FF) Infants, Based on a Linear Discriminant Analysis of Genetic Material Collected from Stool Samples

Gene Name	Function	Fold change (BF/FF)
EPAS1	Transcription factor (TF); cellular response to hypoxia	3.3
NR5A2	TF, encodes liver receptor homologue-1 (LRH-1); development	2.8
NR3C1	Encodes glucocorticoid receptor	5.5
PCDH7	Encodes protocadherin-7; membrane protein	3.9
ITGB2	Encodes integrin beta-2 (CD18); ICAM-1 receptor	2.5
FGF5	Encodes fibroblast growth factor 5; mitogenesis and cell survival	2.0
TJP1	Encodes ZO-1; intercellular tight junctions	2.2
MYB	TF, transcriptional transactivation; proto-oncogene	2.8
EPIM	Syntaxin 2/epimorphin; epithelial cell morphogenesis	2.5
BAD	BCL2-associated agonist of apoptosis	4.0

SOURCE: Chapkin et al., 2010.

plain some of the clinical and epidemiological evidence that has accumulated over the years showing that breast-fed babies' guts develop differently and are less leaky than those of formula-fed babies. For example, the expression of *TJPI*, which encodes ZO-1, an intercellular protein that plays an important role in regulating tight junctions, was also upregulated in the cells from breast-fed babies. Additionally, expression of *NR3C1*, which encodes a glucocorticoid receptor that plays a role in gut differentiation, was fivefold higher in breast-fed than in formula-fed infants.

Using MetaCore, a bioinformatics tool that provides information about function, researchers found that some of the strongest signals were with combinations of genes that encode signaling pathways involved in fundamental pathways of intestinal stem cell proliferation and differentiation, such as WNT and NOTCH.

Donovan suggested that these various gene and gene network classifiers could serve as potential biomarkers for differentiating between breast-fed and formula-fed infants. Also, it would be interesting to see if addition of any of these to infant formula, in the form of a prebiotic or probiotic, would shift the gut microbiota toward the direction of breast-fed infants.

Next, Donovan and her team tested the hypothesis that the integration of infant (host) epithelial cell transcriptome and functionally profiled microbiome can be used to suggest important regulatory pathways of the microbiome affecting intestinal development in the first few months of life (Schwartz et al., 2012). Community-wide microbial gene expression in stool

from the same breast-fed ($N = 6$) and formula-fed ($N = 6$) infants from their earlier work (Chapkin et al., 2010) using established protocols (Poroyko et al., 2010) was evaluated using 454 pyrosequencing of DNA libraries created from stool samples. Taxonomic composition of the metagenome was analyzed with the metagenomics analysis server MG-RAST using similarity to a large nonredundant protein database. Using the same database, the sequence alignments for known microbial metabolic functions were tested against the SEED subsystems.[4] At the phyla level, all formula-fed infants shared the same distinct signature, whereas breast-fed infants were more variable. Three of the breast-fed infants had similar profiles, but the other three "were sort of going to the beat of their own drummer," said Donovan. She noted that all of the formula-fed infants were receiving the same formula but that breast milk composition can be highly variable.

Using established protocols for evaluating community-wide microbial gene expression in stool samples, the team observed a greater total percentage (i.e., percentage of total 16S ribosomal RNA [rRNA]) of Actinobacteria, Proteobacteria, and Bacteroidetes in breast-fed piglets as a group and a greater total percentage of Firmicutes and no Bacteroidetes in formula-fed infants as a group (see Figure 5-1) (Donovan et al., 2012). Donovan remarked that these findings warrant follow-up, given Peter Turnbaugh and colleagues' observation that obesity in mice is associated with a higher Firmicutes:Bacteroidetes ratio. In addition, epidemiological studies have demonstrated that breast-feeding protects against the development of childhood obesity.

Which Human Genes Respond to Bacterial Signals?

An overarching theme of the workshop discussion was the importance and growing interest in understanding not just what bacteria are present in the microbiome, but how those bacteria are signaling in a way that impacts the human host biology. For example, Donovan expressed curiosity about whether differences in microbial gene expression between breast-fed and formula-fed infants impact host gene expression. A comparison of the functional SEED categories of the stool metagenome of breast-fed and formula-fed infants demonstrated a significantly higher proportion of virulence genes in the breast-fed infants. Next, the scientists applied a systematic and statistically rigorous analytic framework for the simultaneous examination of both host and microbial responses to dietary or environmental components in the early neonatal period. Specifically, using canonical correlation analysis,

[4] SEED is an open-source software platform that seeks to curate microbial genomic data into subsystems-based functional annotation (e.g., amino acid metabolism). More information is available online: www.theseed.org (accessed August 28, 2012).

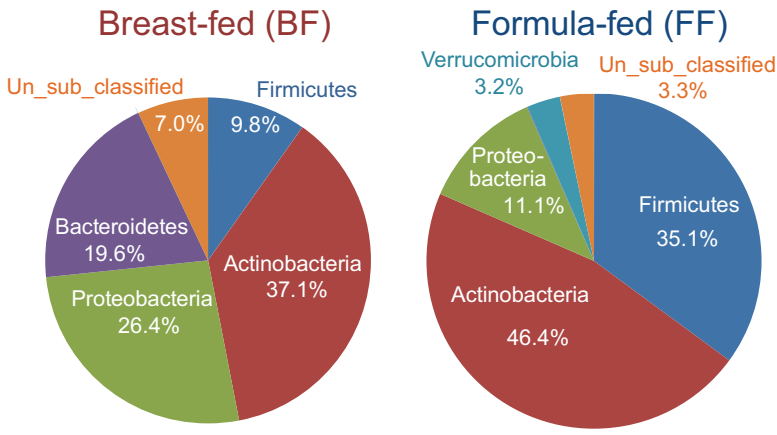

FIGURE 5-1 Results from a study on breast-fed versus formula-fed human infants showing variation in gut microbial composition.
SOURCE: Donovan et al., 2012.

Donovan and her team identified associations between the microbiome virulence genes and 11 host immunity defense genes (*TACR1*, *VAV2*, *ALOX5*, *NDST*, *REL*, *BPILI*, *AOC3*, *KLRF1*, *DUOX2*, *IL1A*, and *SP2*) (Schwartz et al., 2012). Donovan speculated on the potential biomarker usefulness of microbial sequencing, in this case as a way to predict host defense mechanisms. These findings suggest that simultaneously examining the multivariate structure underlying the microbiome and gut transcriptome leverages richer and fuller information content compared to analyses focusing on single datasets (e.g., only host transcriptome data or only microbiome data) and only single variables (e.g., gene-by-gene differential expression testing). The use of canonical correlation analysis can support the formulation of hypothesis-based studies by accurately identifying those genes active in commensal microbiome and host activities (Schwartz et al., 2012).

What Components in the Infant Diet Affect the Intestinal Microbiota?

Nutrients and bioactive components in human milk directly influence the development of the infant's immune system, actively protect the infant from pathogenic infection, and facilitate establishment of the microbiota, the last of which is required to activate the mucosal immune system. Recent data suggest that HMOs contribute to many of these activities (Donovan et al., 2012). Oligosaccharides are the third most predominant component

of milk, after lactose and fat, and up to 200 different structural forms have been identified in human milk (Wu et al., 2010, 2011). Since HMOs are resistant to digestion by the infant and pass into the colon, Donovan described them as the fiber of human milk, a fact that she said hasn't really been appreciated until recently. In the colon, they potentially function in a variety of ways, including as substrates for fermentation and the production of short-chain fatty acids. They can also serve as prebiotics for beneficial bacteria. Donovan referred to David Mills's very elegant data showing that *Bifidobacterium infantis* metabolizes specific HMOs (see the previous section for a more detailed description of work conducted in the Mills laboratory and by Bruce German).

HMO composition is influenced partly by secretor status of the mother and whether she has the 2-fucosyltransferase gene; non-secretor mothers do not produce 2'-fucosyllactose (2'-FL), which is one of the primary HMOs in the milk of secretor mothers. Therefore, Donovan and others are exploring potential predictive associations between HMO composition and infant gut microbiota. Systematic evaluation of the impact of HMO on infant development, however, has been limited by the lack of sufficient quantities of pure HMOs to conduct animal or human feeding studies. However, in the near future, this limitation will be overcome through improved synthetic approaches, opening avenues of investigation into the biology of HMOs. Additionally, the availability of noninvasive methods of assessing outcomes in human infants (Chapkin et al., 2010; Schwartz et al., 2012) and high-throughput methods for measuring HMOs (Wu et al., 2010, 2011) and the infant microbiome (Schwartz et al., 2012) will facilitate our understanding of the role of HMOs in host-microbe interactions in the developing infant (Donovan et al., 2012).

THE RESISTOME AS A DRIVER OF THE MICROBIOME[5]

Food is not the only major driver of the microbiome. So too is the way we raise food, Ellen Silbergeld stated. Most food animals are grown very intensively, including through the use of animal feeds that contain antibiotics. Food and Drug Administration (FDA) data indicate that 80 percent of total antimicrobial production in the United States in 2009 was for use in animal feed.[6] Silbergeld stressed that the use of antibiotics in animal feed is not for veterinary medical purposes; rather, antibiotics are added to feed

[5] This section summarizes the presentation of Ellen Silbergeld.

[6] These values were calculated by the Center for a Livable Future based on data provided by the Food and Drug Administration. For more information, read the posting on its website: http://www.livablefutureblog.com/2010/12/new-fda-numbers-reveal-food-animals-consume-lion%E2%80%99s-share-of-antibiotics (accessed September 19, 2012).

as growth promotants. This is a new phenomenon in the history of antimicrobials and one with significant implications for what Silbergeld referred to as the "resistome."

The Resistome

The term "resistome" was introduced by Wright (2007), who defined it as the collection of all the antibiotic resistance genes and their precursors in the entire microbial community of both pathogenic and nonpathogenic bacteria. Genes within the resistome encode molecular changes that confer phenotypic resistance to both general and specific antibiotic molecules. They are often clustered in cassettes and are transferable by plasmids, creating a pleiotropic efficacy. Multigene cassettes can encode other phenotypes, not just resistance.

An important feature of the resistome is that resistance genes are readily transferred from one bacterial cell to another via horizontal, or lateral, gene transfer (usually via plasmid-mediated transfers but also by conjugation). The classic model of antibiotic resistance describes a population of diverse organisms encountering antibiotic pressure, with some organisms being susceptible and some resistant and with the susceptible organisms dying and the resistant organisms persisting (Sommer and Dantas, 2011). However, that model does not account for the dynamic nature of horizontal gene transfer and the fact that a population of initially susceptible bacteria can accumulate expressible resistance genes over time. Even after a stressor is withdrawn, this system can be permanently altered by the experience. In a study of humans exposed to ciprofloxacin, Dethlefsen and Relman (2011) showed that resistant phenotypes persisted even after exposure ended.

Increasingly, horizontal gene transfer involves not just the sharing of single genes, but also the sharing of cassettes of multiple genes. Silbergeld explained how the extensive use of antimicrobials exerts multiple and repeated pressures on bacterial populations, resulting in sequential acquisition of resistance genes and buildup of multigene cassettes (Canton and Ruiz-Garbajosa, 2011). As empirical evidence of the buildup of transferable multigene cassettes, Silbergeld mentioned U.S. Department of Agriculture (USDA) data showing growth over time of extended multidrug resistance phenotypes of *Escherichia coli* in domestic animals (i.e., chicken, swine, cat, dog, dairy cattle) (Lindsey et al., 2011).

Not only is the resistome accruing more resistance genes, either singly or bundled in multigene cassettes, it also appears to be accumulating networks of preferential horizontal gene transfers, or "cliques" (Skippington and Ragan, 2011). Again, extensive antimicrobial use is exerting selective pressure, in this case for more active networks of horizontal gene transfer.

The bacteria involved in any given network, or clique, are not necessarily in close proximity and may not even share the same ecology.

Silbergeld described the resistome as being analogous to cloud computing, because it is a resource that can be externalized and accessed by various groups of bacteria and because the transfer of resistance genes via horizontal gene transfer is like transferring downloaded bytes of data. She stressed the importance of recognizing that the resistome encompasses both pathogenic and nonpathogenic bacteria and may include most of the bacteria in a specific microbiome.

From the Modern Livestock Farm to Humans: Implications for the Resistome

Selective pressures exerted by extensive antibiotic use abound in the modern livestock farm, which Silbergeld described as an "impressive laboratory for driving microbial evolution." Danzeisen et al. (2011) sampled the microbiomes of chicken ceca after feeding chickens either control feed, feed with monensin (an antibiotic), or feed with virginiamycin (another antibiotic) and detected significant differences in microbiome content (e.g., percentage of total microbes represented by Lachnospiraceae versus Ruminococaceae). However, it is not just the flora that is changing in the face of increased antimicrobial pressure. Researchers are also reporting an increased prevalence of antibiotic-resistant phenotypes in food animals fed antibiotic-containing diets (Looft et al., 2012) (see Figure 5-2).

Importantly, the increased prevalence of resistant phenotypes being observed in the guts of farm animals persists not just in their microbiome but in the resistome at large, mainly because of the practice of land disposal of animal wastes without required pretreatment. For example, Nandi et al. (2004) traced the movement of resistance genes from poultry litter into the soil environment where poultry waste was deposited. Eventually, humans can potentially become exposed to those same resistance genes via several routes.

One way to represent the resistome and the way it transcends, or extends across, all of these different microbiomes, from farm animals to soil bacteria to the human gastrointestinal (GI) tract, is as a nested system of increasing complexity, where components occupy different spaces in the ecosystem and events that occur can eventually impact the human microbiome (Davis et al., 2011). Silbergeld's research group is conducting an ongoing study of the historical ecology of the Chesapeake Bay to see if the appearance of resistance genes in sediment correlates with the introduction of intensive poultry production and the use of antibiotics in poultry feed.

Some experts consider antibiotic-resistant genes to be environmental pollutants that bioaccumulate over time (Martinez, 2009). Although antibiotics are generally not very persistent in the environment, if humans

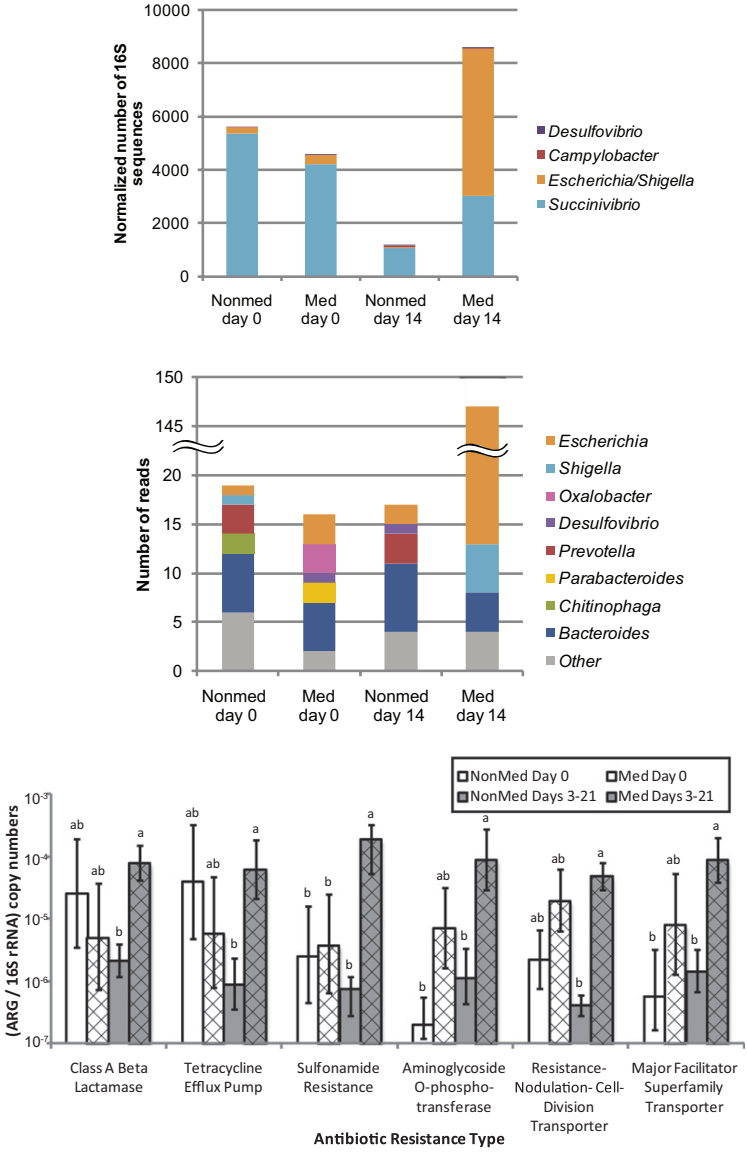

FIGURE 5-2 Results from a study showing a shift in gut microbiota (top figures) and an increased prevalence of antibiotic-resistant phenotypes (bottom figure) in food animals fed antibiotic-containing diets ("GPA feeds").
NOTES: ARG = antibiotic-resistant gene; med = antibiotic-containing diet; nonmed = control. Columns with the same letter are not statistically significant ($p > 0.05$) within each resistance type.
SOURCE: Looft et al., 2012.

continuously add them to the environment—for example, by adding farm animal waste to soil—they can become persistently present, not just in any given microbiome but in the environment at large. Bioaccumulation of antibiotic resistance also occurs through the expansion of microbial populations, and this makes sense evolutionarily, according to Silbergeld. Scientists used to think of antimicrobial resistance as being costly for a microbe to maintain in the absence of antimicrobial pressure, but work by Levin et al. (2000) has shown that in many cases, it is less costly and more efficient for a bacterium to accumulate additional mutations that reduce these costs. This fact, Silbergeld said, may partly explain why resistant bacteria are so persistent even after the antimicrobial stressor is removed. Because of the large number of antimicrobials now in the environment, this may be an efficient evolutionary strategy, as suggested by Martinez (2009).

Silbergeld agreed with Vincent Young, Richard Darveau, and other speakers that the bad-bugs–good-drugs paradigm is too simplistic. Resistant genes can readily travel from one "bug" to another via horizontal gene transfer, either as naked DNA or on cassettes. Even studying the microbiome may not be enough. The challenge is to locate the resistome in space (e.g., Where is it within the microbiome? Where is it in the ecosystem?) and identify where gene transfer and "cross-talk" among microbes occur.

PROBIOTIC MECHANISMS OF ACTION[7]

> *A reader … may be surprised by my recommendation to absorb large quantities of microbes, as a general belief that microbes are harmful. This belief is erroneous. There are many useful microbes, amongst which the lactic bacilli have an honorable place.* —Elie Metchnikov (1907)

While the fundamental conceptual framework for probiotics was laid out in the early 20th century by Elie Metchnikov (1845-1916), many of today's scientists use the Food and Agriculture Organization-World Health Organization (FAO-WHO) (2002) definition as their working definition. FAO-WHO (2002) defines probiotics as living microorganisms that when administered in adequate amounts confer a health benefit on their host. James Versalovic called attention to three features of the FAO-WHO definition. First, probiotics are living, viable microorganisms. Second, for a microorganism to be considered a probiotic, it has to be administered in adequate amounts. Third, a probiotic must confer some kind of health benefit. For Versalovic, the question is, How do they confer that health benefit? Or, as he put it, how do they "optimize the functioning of our physiology"? Versalovic listed several potential mechanisms of action: stimulation of

[7] This section summarizes the presentation of James Versalovic.

immunity, suppression of immunity (both innate and adaptive immunity), promotion of intestinal epithelial cell development and migration, alteration of microbiome composition and function, enhanced recovery from infection, and antimicrobial functions.

Stimulation of Immunity

Probiotics can directly stimulate both adaptive and innate immunity (Thomas and Versalovic, 2010). They can also indirectly impact host immunity by enhancing host ability to digest and absorb nutrients that have an impact on the immune system. Both the direct and the indirect effects have been described in detail in a number of studies published over the past couple of decades. For example, Yamanaka et al. (2003) reported that gut bacteria drive Peyer's patch[8] development in rats, with germ-free rats having defective and immature lymphoid follicles and conventionalized rats having very mature gut lymphoid tissue. Indeed, it has been proposed that a key function of the gut microbiome might be to serve as a "treadmill" for the host immune system (Madara, 2004). By "tickling" Toll-like receptors and other receptors and signaling pathways that build host immunity, gut microbes might be keeping the host immune system "finely tuned and fit" and preparing the immune system for new challenges (e.g., antibiotic exposure, changes in diet). Evidence supports this hypothesis. As just one example, Prescott et al. (2008) reported that pregnant women who took either a *Bifidobacterium* or *Lactobacillus* probiotic had significantly elevated levels of interferon-gamma levels in their cord blood and that maternal probiotics may enhance interferon-gamma production in neonates. Interferon-gamma production has been associated with protection against allergic disease early in life (Macaubas et al., 2003). Prescott et al. (2008) also showed a significant effect of maternal probiotics on antibody production in breast milk. Breast milk immunoglobulin A (IgA) levels were significantly elevated at both 1 week and 3 months of age in the children of women who took either a *B. lactis* or *L. rhamnosus* probiotic. Although the difference in IgA levels between the children of the experimental and control women disappeared by 6 months of age, infants fed breast milk with elevated IgA levels early on may be receiving a critical head start in gaining passive immunity.

Suppression of Immunity

Evidence suggests that probiotics don't just stimulate immunity, they also suppress it. Versalovic's research group has conducted mouse model

[8] Peyer's patches are aggregates of lymphoid follicles located in the epithelium of the small intestine.

studies showing that the probiotic *Lactobacillus reuteri* can suppress proinflammatory cytokines by secreting very small soluble factor(s) (Thomas and Versalovic, 2010).[9] Using nuclear magnetic resonance (NMR) and other advanced metabolomic technologies, Thomas et al. (2012) identified one of these soluble factors as histamine and reported a correlation between elevated levels of bacterial-derived histamine and potent tumor necrosis factor (TNF) suppression in human monocytoid cells. According to Versalovic, the correlation has been found in a variety of other cell lines as well. As far as mechanism, Thomas et al. (2012) showed in vitro that bacterial-derived histamine suppresses TNF production by binding to histamine type 2 (H_2) receptors and blocking mitogen-activated protein (MAP) kinase signaling pathways. "This was clearly surprising that we would have histamine as an immunoregulatory molecule," Versalovic said. "But the real punch line is not the histamine. It's histidine."

Histidine is a dietary component that serves as the chemical precursor to histamine. Several research groups have identified three genes involved in histidine-to-histamine biosynthesis: *hdcA*, *hdcB*, and *hdcP* (Copeland et al., 1989; Martin et al., 2005; Trip et al., 2011). Versalovic's team generated single-gene knockouts of the probiotic strain *L. reuteri* ATCC 6475 for all three genes and showed in vitro that knocking out any one of the three genes eliminates a large chunk—40 percent or more—of bacterial TNF inhibitory activity (Thomas et al., 2012). The research group is testing the single-gene knockouts in vivo. Unpublished data indicate that *L. reuteri* 6475 *hdcA* knockouts administered to mice via orogastric gavage cause a diminished ability to attenuate colitis following a TNBS (2,4,6-trinitrobenzenesulfonic acid) challenge.[10] By contrast, when mice are administered wild-type probiotic, their disease state ameliorates. The next step, Versalovic noted, is to add more histidine to the diet via modified mouse chow and see if that enhances the anti-inflammatory effect of the wild-type probiotic.

Histamine is "a large part of the story," Versalovic said, but it's not the whole story. The "other part of the punch line" is that histamine operates as an immunomodulatory compound only in the presence of the H_2 receptor. The H_1 receptor, by contrast, triggers a proinflammatory response. So the effect of histamine—and histidine—in the diet is dependent on the relative distribution of H_2 versus H_1 receptors. Versalovic's team is exploring the biogeography of H_2 receptors and ways in which they can be enhanced.

[9] Versalovic noted that most of his research on probiotics is with *L. reuteri*. Not only is it a widely used probiotic, it is also an indigenous member of the human microbiome (Reuter, 2001).

[10] The TNBS challenge is standard protocol for inducing murine colitis, that is, the mouse equivalent of human inflammatory bowel disease.

Other research groups are thinking along the same line, that is, that a dietary amino acid, in this case histidine (specifically, L-histidine), can be converted by gut bacteria into another compound with immunomodulatory effects (Andou et al., 2009; Blumberg and Strober, 2001).

Promotion of the Intestinal Epithelium

In addition to their impact on host immunity, probiotics also impact development of the intestinal epithelium. For example, Preidis et al. (2012a,b) demonstrated that gut bacteria can prevent or ameliorate *Rotavirus*-associated acute gastroenteritis in a neonatal mouse model. Administering two different strains of *Lactobacillus reuteri* led to about a 1-day reduction in disease duration, which is a significant amount of time in the case of acute infection. One *L. reuteri* strain in particular had a dramatic effect on maturation of epithelial cell walls. It is not clear what the signals are, that is, how these microbes are enhancing the ability of the epithelium to differentiate. Data from expression profiling studies suggest that *L. reuteri* may be promoting the sloughing of *Rotavirus*-infected cells by altering the actin cytoskeleton and weakening attachments of the basement membrane, thereby increasing epithelial cell migration and turnover.

Other Mechanisms of Action

In addition to host immunity and intestinal epithelium development, probiotics can also influence human health and disease by enhancing microbiome diversity or, more compellingly, by changing microbiome gene expression. They can also impact antimicrobial production by the microbiome (e.g., *L. reuteri* produces reuterin). Versalovic suggested that a probiotic antimicrobial strategy could replace the widespread use of antibiotics in animal farms.

The Future

Rapidly advancing knowledge of the microbiome could be used to create "designer strains" of probiotics that enhance health or prevent disease (Preidis and Versalovic, 2009). Alternatively, it might be possible to select natural strains of probiotics that make foods even more effective and functional than they already are in terms of health maintenance and disease prevention.

PREBIOTIC MECHANISMS OF ACTION[11]

Food ingredients and novel compounds are increasingly being examined for their ability to do more than provide nutrition, according to George Fahey. Most of this expanding research activity is focused on health promotion or disease reduction. At the top of the list of food ingredients being studied for nonnutrition activity are the nondigestible oligosaccharides (NDOs). Nutritionally, NDOs are known mostly for their low caloric value and ability to enhance mineral absorption, but they are also becoming known for their potential to lower the risk of infections and diarrhea, modulate the immune system, and modulate the microbiota. At the beginning of the prebiotic era, in the mid-1990s (Gibson and Roberfroid, 1995), scientists spent a great deal of time in particular thinking about how NDOs might be used to increase the presence of beneficial bifidobacteria (members of the genus *Bifidobacterium*) and lactobacilli (members of the genus *Lactobacillus*) in the microbiota while decreasing the presence of pathogenic bacteria. Many NDOs have the ability to alter the composition of the colonic microbiota in a positive manner, thus satisfying a key criterion for what defines a prebiotic—the selective stimulation of growth and/or activity of those bacteria that contribute to colonic and host health.

What Are the Major Dietary Sources of Prebiotics?

There are several well-established major dietary sources of prebiotics, primarily fructins (including chicory root extract, inulin, oligofructose, and short-chain fructooligosaccharides). Two other major dietary sources of prebiotics are the galactooligosaccharides and the stool softener lactulose.

There is a long list of potential prebiotic candidates, including soybean oligosaccharides, glucooligosaccharides, cyclodextrins, gentiooligosaccharides, oligodextrans, glucorinic acid, pectic oligosaccharides, isomaltooligosaccharides, lactosucrose, xylooligosaccharides, human milk oligosaccharides, mannanoligosaccharides (yeast cell wall), lactose, resistant starch and derivatives, oligosaccharides from melobiose, *N*-acetylchitooligosaccharides, polydextrose, sugar alcohols, and konjac glucomannan. These are widely variable types of compounds, Fahey noted. Several are natural ingredients (e.g., soybean oligosaccharides), and several are widely used in both human and animal diets (e.g., yeast cell wall, which is very rich in mannanoligosaccharides). Some are very simple from the point of view of chemical composition (e.g., glucooligosaccharides); others are "really strange," according to Fahey (e.g., *N*-acetylchitooligosaccharide). These and other candidates are considered "potential" because research on their prebiotic characteristics is incomplete,

[11] This section summarizes the presentation of George Fahey.

and they have not been shown to meet all of the specific requirements of the current working definition of a prebiotic.

Prebiotics and prebiotic candidates are produced from a variety of raw materials, such as chicory, artichoke, beet, cow's milk, starch, and soybean (Mussatto and Mancilha, 2007). Production typically involves extracting an intermediate product (e.g., inulin from chicory and artichoke, sucrose from beet, lactose from cow's milk, soluble starch from starch, soybean whey and xylan from soybean) and then using one of several processes (i.e., hydrolysis, transglycosylation, isomerization, extraction) to isolate the actual prebiotic.

How Do Prebiotics Modify the Composition of the Microbiota?

The effect of a prebiotic (or potential prebiotic) on bacterial growth depends on the type of prebiotic (or potential prebiotic) ingested. In a study on the effect of resistant starch on fecal microbiota in 10 healthy human volunteers, Martinez et al. (2010) observed significant changes in the relative proportions of various bacterial taxa depending on the type of resistant starch ingested. Researchers fed the volunteers three types of crackers in a 17-week double-blind crossover study: RS2 (crackers made with Hi-Maize 260, a resistant starch 2), RS4 (crackers made with a chemically modified, phosphorylated, cross-linked type 4 resistant starch, Fibersym RW), and native wheat starch (the control). All subjects consumed 33 grams of resistant starch per day. Consumption of RS4 increased the proportion of phylum Firmicutes and decreased the proportions of Bacteroidetes and Actinobacteria relative to the control and, in the case of Firmicutes and Actinobacteria, relative to the consumption of resistant starch 2 as well. At the family level, researchers observed increased proportions of Bifidobacteriaceae and Porphyromonadaceae in the RS4 treatment and decreased proportions of Ruminococcaceae and Erysipelotrichaceae relative to the control. At the genus level, they observed increased proportions of *Parabacteroides* and *Bifidobacterium* and decreased proportions of *Faecalibacterium* and *Dorea* in the RS4 treatment compared to the control.

As another example of the variable effects of different types of prebiotics, in a randomized, double-blind, placebo-controlled, crossover study of 20 healthy men between 21 and 28 years of age, Hooda et al. (2012) observed significant differences in the proportion of bacterial genera detected in feces depending on which of three types of fiber were consumed. Researchers fed the volunteers three fiber bars per day, with each bar containing either no supplemental fiber or 7 grams of either polydextrose (PDX) or soluble corn fiber (SCF). They observed large and significant increases in the

proportions of *Faecalibacterium*, *Phascolarctobacterium*, and *Dialister* in feces following consumption of both PDX and SCF and a small but statistically significant increase in *Lactobacillus* following consumption of SCF.

The extent to which a prebiotic (or potential prebiotic) stimulates microbial growth depends not just on the type of substance ingested, but also on its dietary concentration. In a 16-week study of galactooligosaccharides (GalOS) in 18 healthy human volunteers between 19 and 50 years of age, Davis et al. (2010) reported two major findings. First, based on culture enumeration, the concentration of *Bifidobacterium* in feces increased significantly among volunteers who were fed 5 or 10 grams of GalOS per day, compared to baseline. There was no significant increase in *Bifidobacterium* in the feces of individuals fed either 0 or 2.5 grams of GalOS per day. Second, individuals fed 10 grams of GalOS per day also had significantly more total anaerobes in their feces compared to baseline. None of the other treatment groups showed a change in total anaerobe concentration. Using quantitative real-time PCR (qRT-PCR) to measure the bifidogenic effects of GalOS, again the researchers found a significant increase in bifidogenic activity among individuals fed 5 or 10 grams of GalOS per day, but not among individuals fed 0 or 2.5 grams per day. With respect to which bacteria are affected by GalOS consumption, Davis and colleagues (2010) pyrosequenced the V1-V3 region of 16S ribosomal DNA (rDNA) and found that GalOS consumption did not impact the diversity of fecal microbes but did impact the relative proportions of bacterial taxa at the phylum, family, genus, and species levels. For example, consumption of 10 grams of GalOS per day increased the proportion of the family Bifidobacteriaceae from 1.56 percent at baseline to 6.14 percent. Within that family, consumption of 10 grams of GalOS per day increased the proportion of *Bifidobacterium* from 1.28 percent at baseline to 5.20 percent.

Fahey referred to previous speakers' comments on the considerable variability that exists among individuals with respect to how their microbiota respond to dietary intervention. Not surprisingly, Davis et al. (2010) observed highly variable responses among individuals at the phylum, family, genus, and species levels. Fahey recognized the limitations that individual variability sets up for a study based on a sample size of 18, but asserted that 18 is a manageable number for such an intensive study.

Sometimes prebiotics are used as supplements at very low levels, even though much of the murine research on inulin, for example, involves daily administration of the human equivalent of 30 to 35 grams of fermentable substrate, which is within the range of the dietary reference intake (DRI)

for men.[12] Human studies show variable effects depending on dietary concentration.

In addition to the type of prebiotic or potential prebiotic ingested and dose, a multitude of other factors influence the way a prebiotic impacts the GI microbiota, including gastric emptying time, intestinal transit time, nutrient digestibility, fecal bulk and frequency of defecation, short-chain fatty acid (SCFA) production, intestinal morphology, gut immune modulation, and the GI microbiota itself.

Are Prebiotics Effective in Achieving Host Health Benefits?

Much of the research on prebiotics is in healthy individuals. Many studies have shown significant increases in the so-called beneficial microbes (i.e., bifidobacteria and lactobacilli) following consumption of GalOS, inulin, and other prebiotics—*in healthy individuals*. Researchers have also reported increases in butyrate producers (e.g., *Eubacterium*, *Faecalibacterium*, *Roseburia*) following consumption of resistant starch, polydextrose, soluble corn fiber, and other prebiotics—again, in healthy individuals. Fahey urged more studies on diseased populations, given that many microbes are associated with disease. For example, inflammatory bowel disease conditions are known to be associated with a decreased proportion of *Faecalibacterium*. Studies have shown that SCF, PDX, inulin, fructooligosaccharides, pea fiber, and other prebiotics or potential prebiotics can impact *Faecalibacterium*. The question remains, Do those same prebiotics or potential prebiotics alleviate inflammatory bowel disease conditions via their impact on *Faecalibacterium*?

Fahey also urged more consideration of how prebiotics impact microbial metabolites, especially butyrate and other SFCAs. Studies have shown that inulin, fructooligosaccharides, and GalOS can increase SFCA levels, but what impact do prebiotics have on the toxic end products of fermentation such as ammonia, phenols, and indoles? Hooda and colleagues (2012) measured some of those toxic end products as part of a larger microbe-health index principal component analysis and found that Lachnospiraceae, Lactobacillaceae, and Veillonellaceae were all negatively correlated with ammonia, phenols, and indoles. Additionally, Lachnospiraceae and Lactobacillaceae were positively correlated with total SCFA, and Veillonellaceae was positively correlated with fiber intake. Veillonellaceae was also negatively correlated with total branched-chain fatty acids (BCFAs).

As an example of a prebiotic study that both used a disease model and

[12] The DRI for total fiber (combination of dietary fiber and functional fiber) for men is 31 grams per day for the 9- to 13-year age group, 38 grams per day for the 14- to 50-year age group, and 30 grams per day for men aged 51 and older (IOM, 2002).

examined metabolic outcome, Everard et al. (2011) fed *ob/ob* mice either a control diet or a diet supplemented with oligofructose for 5 weeks. At the end of the 5 weeks, they sequenced the V1-V3 region of 16S rDNA and conducted various glucose metabolism tests. The results of the 16S rDNA analysis showed phylum-level increases in Bacteroidetes, Actinobacteria, and Proteobacteria and a decrease in Firmicutes. At the family level, they detected Bifidobacteriaceae in the prebiotic-fed group, but not in the controls. At the genus level, again they detected *Bifidobacterium* only in the prebiotic-fed mice. The glucose tolerance testing showed several positive metabolic outcomes associated with prebiotic consumption: lower fasting glycemia level, improved glucose tolerance, decreased fat-to-muscle mass ratio, decreased plasma triglycerides, improved gut barrier function, lower plasma lipopolysaccharide (LPS) concentrations, and reduced expression of oxidative stress and inflammatory markers.

Potential Ways to Advance the Field of Prebiotics

In addition to more research on diseased populations and on microbial metabolites, Fahey suggested several other ways to advance the field of prebiotics. First, conduct more compositional analyses of potential prebiotics. "We do too little of that," he said. Knowing the monomeric composition, chain length, linkages, branching, side chains, and other features of the structural composition of a prebiotic can help to interpret the biological data. Second, examine prebiotic activities in natural foods, such as soybean products, beet fiber, and whole grains and their co-products. Third, continue to look beyond the bifidobacteria. Fourth, study microbiome–health index relationships, à la Hooda et al. (2012).

TRANSLATION OF PROBIOTIC SCIENCE INTO PROBIOTIC FOODS[13]

How can knowledge about the microbiome influence the design of healthy food, including probiotic foods? Scientists know that probiotics can impact the microbiome, both directly and indirectly, as James Versalovic described during his presentation (O'Toole and Cooney, 2008). They also know that probiotics can impact health. What they don't know, according to Mary Ellen Sanders, is whether probiotic impacts on the microbiome are directly responsible for the observed human health benefits. Most studies that correlate microbiome changes and human health benefit do not reveal anything about causality. So the question remains, Do probiotics have a beneficial effect on health through their direct or indirect actions on the

[13] This section summarizes the presentation of Mary Ellen Sanders.

microbiome? "I would say the answer to that is likely yes," Sanders said, but such causality needs to be confirmed. Sanders provided an overview of demonstrated effects of probiotics on the microbiome and on health and scientific challenges to translating this knowledge into probiotic foods.

Impact of Probiotics on the Microbiome

There is plentiful evidence of the effects of probiotics on the microbiome, especially intestinal microbiota (Sanders, 2011). The most common impact of probiotics on the intestinal microbiota, or more accurately the fecal microbiota, is an increase in the particular strain that the test individuals have been fed. Probiotics expand across a wide taxonomic range and even include yeast (i.e., *Saccharomyces*). However, researchers usually feed their test subjects probiotics that they know will survive intestinal transit. Another common observation is changes in metabolic parameters that can be either local or pan-organismal. These changes can be observed not just in the feces or colon, but also in the urine and in other tissues. Probiotics have also been observed to impact pathogens, as evidenced by changes in the infectivity and toxicity of pathogens. Researchers have also observed changes in the community structure of indigenous microbiota, although results vary among probiotics and among studies. For example, different probiotics have been shown to increase community evenness, functional redundancy, and specific types of potentially beneficial bacteria. Finally, and one of the more interesting effects in Sanders's opinion, is that probiotics have been shown to encourage homeostasis, or stability, of the microbiota. It has been hypothesized that maintaining the microbiota in an "even state" has beneficial physiological effects; microbiota that maintain a certain evenness, or stability, may be able to rebound more quickly when perturbed by an antibiotic or other stressor (Sanders, 2011).

When considering beneficial effects of probiotics on the microbiome, it must be remembered that experts have yet to reach consensus on what a healthy microbiome looks like. This fact makes it difficult to know which probiotic effects on the microbiome are likely to translate into health benefits for the host.

Demonstrated Health Benefits of Probiotics

The demonstrated health benefits of probiotics go beyond the gut. Researchers have investigated a wide range of end points, including oral microbiology (e.g., dental caries), allergies (e.g., atopic dermatitis, asthma), vaginal infections, mental function, skin microbiology, acute upper respiratory tract infections, and various global end points (e.g., growth parameters of undernourished children, reduced absences from work or day care,

quality-of-life indicators). Even within the gut, a wide range of end points have been tested. These include acute diarrhea, antibiotic-associated diarrhea, travelers' diarrhea, *C. difficile* infection, lactose digestion, irritable bowel syndrome (IBS) symptoms, colic, inflammatory bowel conditions, and gut pain sensation.

In Sanders's opinion, the field is embracing evidence-based approaches to conclusions on the health effects of probiotics, as demonstrated by the many systematic reviews and meta-analyses that have been published. As of November 2011 (the time of this IOM workshop), Sanders had identified 66 such reviews in the scientific literature. The end points cover a very broad range of body sites and conditions, including NEC; infant growth; persistent diarrhea; radiation-induced diarrhea; antibiotic-associated diarrhea; travelers' diarrhea; *H. pylori*; Crohn's disease, ulcerative colitis, and pouchitis; IBS; digestive symptoms; allergy; critical care or hospital infections; bacterial vaginosis; acute respiratory tract infections; and safety. Sanders noted that most of these end points are drug (i.e., can cure, treat, mitigate, or prevent disease) and not food end points. A common misperception is that a probiotic dose needs to be at least 10^9 in order to be effective, Sanders noted, but there is no single best minimum dose. Rather, whatever dose was used in the human study that showed a significant positive effect should be the minimum dose for that probiotic (Savino et al., 2007; Whorwell et al., 2006).

Significance of Strain Specificity

For the past 20 years, researchers have been emphasizing the importance of strain specificity. Plentiful evidence from animal models shows this to be the case, according to Sanders. The effectiveness of one strain of a species does not necessarily mean that other strains are equally effective. In a comparison of five different commercial probiotic preparations, Canani et al. (2007) observed variable effects on the duration of diarrhea in children, with only two of the commercial preparations demonstrating effectiveness (see Table 5-2). Sanders suggested that part of the reason that commercial products labeled as probiotic do not necessarily have similar effects could be that variable combinations of strains are used.

The challenge of strain specificity raises the question, Are there shared effects among phylogenetically related strains? For example, almost all studies on the two yogurt-containing probiotics *Streptococcus thermophilus* and *Lactobacillus bulgaricus* have demonstrated reduced lactose maldigestion in people who are lactose-intolerant. Regardless of strain, all yogurts containing at least 10^8 live starter *S. thermophilus* and *L. bulgaricus* per gram can bear the claim that the product will improve lactose digestion in individuals with lactose maldigestion (EFSA, 2010). Sanders asked, Could

TABLE 5-2 Comparison of Five Commercial Probiotic Preparations on Duration of Diarrhea in Children

Treatment	Median (IQR) Duration (hours)	Estimated Difference (95% CI)	*P*-Value
Oral rehydration solution alone	115.5 (95.2-127)	—	—
Lactobacillus casei subp *rhamnosus* GG	78.5 (56.5-104.5)	–32 (–41 to –23)	<0.001
Saccharomyces boulardil	105.0 (90-104.5)	–5 (–13 to 5)	0.38
Bacillus clausii	118.0 (95.2-128.7)	1 (–7 to 8)	0.76
L. delbrueckii var. *bulgaricus, L. acidophilus, Streptococcus thermophilus, B. bifidum*	70.0 (49-101)	–37 (–47 to –25)	<0.001
Enterococcus faecium SF 68	115.0 (89-144)	2 (–5 to 11)	0.61

NOTE: *N* = 571 children aged 3-36 months presenting with acute diarrhea; 5-day treatment period. CI = confidence interval; IQR = interquartile range.
SOURCE: Canani et al., 2007.

other general claims be made about groups of phylogenetically related strains? For example, can multiple strains of *Bifidobacterium* improve digestive comfort, or might multiple strains of *Lactobacillus* increase short-chain fatty acids in the colon? To be convincing, these more general claims will require demonstrating that multiple strains of the same taxonomic group have the same effect and either that a common mechanism of action among the strains mediates this effect or that different mechanisms of action among the strains result in the same effect. Sanders was hopeful that "there may be a time in this field when enough accumulated data are present that we are able to say that although certain activities are definitely linked to certain strains, others do seem to be more broadly attributable to broader microbiological categories or phylogenetic types."

Challenges to Translating Probiotic Science into Probiotic Foods

While the plethora of probiotic products on the market seems to suggest a lack of any barriers to the development of probiotic foods, in fact there are many scientific, regulatory, technological, and marketing challenges. Sanders elaborated on a couple of key scientific and regulatory challenges.

In addition to strain specificity, another major scientific challenge is that the magnitude of the demonstrated effect must be meaningful. Sanders

remarked that if experimental study does not demonstrate a meaningful effect, nothing of interest has really been demonstrated. This raises the question, What is a meaningful magnitude of effect? She asked members of the workshop audience whether they considered an intervention that decreases absences from school by half a day per year enough of a magnitude of effect to consider the intervention worthwhile. Few audience members nodded yes. Yet, that is exactly the magnitude of effect demonstrated in a study on hand-washing by children—and it was enough of an effect to justify a national hand-washing campaign by the Centers for Disease Control and Prevention. Using the same end point—missed school days—Leyer et al. (2009) showed that a 6-month course of *L. acidophilus* NCFM/*B. animalis* Bi-07 probiotic resulted in more than 1 fewer missed school days per child. That is double the effect of hand-washing, Sanders noted. "Sometimes in the probiotic field we kind of beat ourselves up because we don't have these overwhelmingly huge magnitudes of effect," she said. "But maybe we don't need them."

Yet another major scientific challenge is that not all studies demonstrate the same effects. Mixed results reflect the considerable individual-level variation in microbiota that has been demonstrated many times (Candela et al., 2010). They also reflect the prevalence of underpowered, small-*N* probiotic studies. An underpowered study providing no evidence of an effect is very different from a sufficiently powered study providing evidence of no effect. Sanders said, "We need to be able to distinguish that and possibly quit running underpowered studies."

In addition to these scientific challenges, Sanders suggested that new regulatory challenges may end up discouraging future probiotic research. In October 2010, FDA issued a guidance on determining when human research studies require Investigational New Drug (IND) applications (FDA, 2010). Sanders interpreted the guidance to mean that any human study on the cure, treatment, mitigation, or prevention of disease or on the structure or function of the body is a drug study and therefore can only be conducted with an IND. "If it's ever finalized in this form," she said, "it will have a chilling effect on research" in probiotics.

In conclusion, Sanders touched on another regulatory challenge that would be explored in depth by subsequent workshop speakers (see Chapter 6), that is, the current regulatory framework for product claims. Probiotic products sitting side by side on the store shelf can be very different regarding both content and scientific evidence for safety and efficacy, but allowable information on claims doesn't enable consumers and health care professionals to differentiate among products. Sanders mentioned Proctor and Gamble's Align. The claim is that Align builds and supports a healthy digestive system, but the scientific evidence is for improved symptoms associated with irritable bowel syndrome (O'Mahony et al., 2005; Whorwell

et al., 2006). The range of allowed claims, even with documented evidence, is too narrow. Sanders commented that consumers and health care providers should be provided with truthful information so that they can make informed choices about probiotics.

DEVELOPING DELIVERY SYSTEMS[14]

Experiments within a well-controlled laboratory or clinical setting may indicate that a particular bacterium is a highly effective probiotic, but in reality the probiotic may not be effective if its bioactivity is lost before it is able to confer any health benefit. Many studies have shown that probiotic bacteria lose their activity over time if they are placed in foods that have not been correctly designed to accommodate those bacteria, according to David Julian McClements. There has been a dramatic increase in probiotic viability studies over the past decade, with many studies showing appreciable reductions in probiotic viability during food storage or during transit through the human GI tract. For example, Priya et al. (2011) reported a 10^8- to 10^9-fold decrease in the number of viable probiotic organisms by the time they reached the small intestine.

Delivery System Design

McClements's research program revolves around the design of encapsulation systems, that is, structured delivery systems that encapsulate, protect, and deliver bioactive compounds to an appropriate site of action within the GI tract. While most of his work is with nutraceuticals, he asserted that the same systems are amenable to utilization for the encapsulation and delivery of live bacteria. In the previously mentioned Priya et al. (2011) study, in addition to researchers observing a dramatic decrease in the number of viable bacteria reaching the colon, they also observed substantial improvement upon encapsulating the bacteria in a multilayer polymer coating (see Figure 5-3).

McClements noted two key considerations to keep in mind when designing delivery systems for probiotics. First, whether a probiotic delivery system will work or not depends on the strain of bacteria, the nature of the delivery system, and the kind of food in which the bacteria is being delivered. He remarked that the tremendous variability observed in the results of probiotic viability studies reflects variation in these factors. Second, foods are low-profit-margin materials. The food industry wants the simplest, cheapest, and most robust solutions to any given problem. Yet, because they are difficult to make and require complicated processing operations,

[14] This section summarizes the presentation of David Julian McClements.

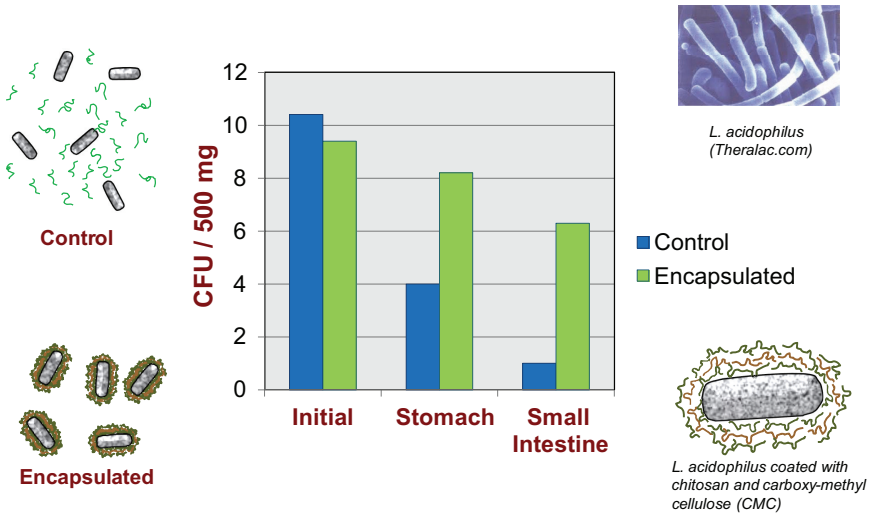

FIGURE 5-3 Results showing increased viability of encapsulated probiotics, relative to non-encapsulated probiotics, as they pass through the human GI tract.
NOTE: CFU = colony-forming unit.
SOURCE: Priya et al., 2011.

delivery systems are often expensive. There are other options for protection, such as controlling food matrix properties (e.g., nutrients, pH, ionic strength, temperature, oxygen, other properties that affect bacterial survival) and selecting resistant microbial strains (e.g., bile- and acid-resistant strains, strains resistant to some of the other stressors to which bacteria are likely to be exposed). Third, any potential delivery system used should not adversely affect the desirable quality attributes of a food, such as appearance, taste, texture, and shelf life, because this will affect the likelihood that an individual will continue to purchase and consume the food.

Encapsulation Technology

In many respects, McClements noted, developing delivery systems for probiotics is similar to what drug manufacturers do when they develop delivery systems for drugs. However, the challenge is even greater because of greater constraints on the types of components that can be used with foods and the complexity of the food matrix. A drug can be placed in a capsule, pill, or syrup, but a probiotic, if it is going to be consumed regularly, needs to be placed in a food matrix in such a way that it does not adversely affect the appearance, taste, texture, or stability (shelf life) of the food, according to McClements. Plus, food products encounter a series of

different stressors during manufacturing, storage, transport, and preparation (e.g., thermal processing, chilling, freezing, dehydration), any of which could affect probiotic viability.

Food delivery system design has been driven in part by the nanotechnology revolution, which has provided new tools that can be used to create molecular and colloidal structures to encapsulate bacteria, protect them from the various challenges they are likely to encounter in food and as they pass through the GI tract, and release the encapsulated bacteria at specific sites in the GI tract. The three most common structural designs are embedding, coating, and a hybrid embedding-coating approach. Embedding involves trapping the probiotic within some sort of solid or liquid matrix made of proteins, polysaccharides, or other components. Coating involves covering the probiotic with one or more layers of dietary fibers, proteins, or other substances. A hybrid embedding-coating approach involves trapping the bacteria within a matrix and then coating the matrix.

Encapsulation technologies are different for dry foods (e.g., cereals, powders, breads) versus wet foods (e.g., beverages, yogurts). Dried products are typically encapsulated using spray drying, yielding 50 micrometer dried powder microencapsulated probiotics. The particles dissolve when exposed to water, releasing the probiotic. Spray drying protects probiotics during food storage but not upon exposure to the human body. A variety of different technologies can be used for wet product encapsulation, including coacervation, bead formation, emulsion formation, and coating. Bead formation involves mixing the probiotics with a polymer solution (such as alginate) and then dripping the mixture into a gelling solution (such as calcium chloride) to form beads with probiotics encapsulated inside. Coacervation involves mixing the probiotics with a mixture of positive and negative polymers to form a hydrogel bead with probiotics trapped inside. Emulsion formation involves using a water-and-oil emulsion to make filled biopolymer particles with the probiotic contained inside. Finally, coating methods involve coating a negatively charged bacterium with a positively charged polymer (monolayer) or a series of positive and negatively charged polymers (multilayer). The advantage of these three technologies is that they maintain their structure when diluted. Unlike dried encapsulation systems, they do not fall apart upon exposure to the human body and can be designed to maintain their viability until they reach a specific region of the GI tract.

Controlling probiotic viability is a challenge. The delivery system has to be designed to withstand the variable challenges it encounters along the length of the GI tract, such as high acidity, lipase activity, antimicrobial activity, and oxygen levels. The stomach is arguably the harshest environment, with a pH typically between 1 and 3, and with enzymes that can break down the delivery system or attack the probiotic (e.g., lipases, proteases),

bile salts, and other stressors. To overcome these challenges, researchers have devised many ways to manipulate delivery system properties to ensure that a system remains viable along the length of the GI tract. Viability is influenced by different characteristics of encapsulation systems: particle size—larger particles are usually more stable during transit through the GI tract; composition—the digestibility of the matrix components determines their response; nutritional profile—the type of nutrients present within a matrix may influence probiotic viability; physical state—solid particles are often more stable than liquid ones; permeability—the pore size of matrices can be changed so that digestive enzymes, bile salts, and other stressors cannot access the probiotic; and environmental responsiveness (e.g., microencapsulated probiotics can be designed to swell or shrink under different pH conditions or ionic strengths). Changing the electrical charge on the delivery system particles can impact where in the GI tract the encapsulated probiotic is likely to attach (e.g., on the mucin layer in a certain region of the GI tract).

Once a system has been designed and developed, it is tested in vitro, which usually involves simulating the mouth, stomach, small intestine, and colon by controlling pH and the types of enzymes and minerals present. In vitro testing can be used to screen different types of delivery systems or various alterations in a delivery system (e.g., Chavarri et al., 2010; Priya et al., 2011). Eventually the system needs to be tested in vivo by using animal and human studies, according to McClements.

Challenges and Opportunities

In conclusion, McClements stated that there is great potential for delivery systems to protect probiotics in foods and within the body. Probiotics that otherwise might not survive in foods under normal conditions or in the human body might need the protection that encapsulation affords. However, in addition to being effective, delivery systems must be economical (i.e., many of the technologies that have been tested in vitro are too expensive for commercial application), practical (e.g., they have to be constructed from food-grade materials), and without any potentially adverse effects.

While several encapsulation systems have been shown to be effective in vitro, a major challenge to translating these results into commercial products is the non–food-grade nature of some of the materials used. The encapsulated probiotics tested in both Priya et al. (2011) and Chavarri et al. (2010) were engineered with a chitosan coating. Not only is chitosan not food-grade, it is also antimicrobial, which could have adverse effects. Sensory quality poses another challenge. The particles tested in Chavarri et al. (2010) were in the millimeter size range, which is too large to incorporate

into a food matrix. The mouth detects as undesirable (in texture) anything larger than about 50 micrometers.

HOW THE MICROBIOME REVOLUTION FUELS FUNCTIONAL FOOD RESEARCH[15]

The main mission of Danone is to help people build and preserve their health capital through food, according to Johan van Hylckama Vlieg. Danone offers consumers products that serve all life stages, from babies (i.e., foods focused on infant nutrition) to older adults (including foods for people with specific nutritional requirements, also referred to as "medical nutrition").

However, Danone—and the food industry at large—is up against some new challenges, not the least of which is a changing demographic context. In 2013, five countries will represent 47 percent of the global population and 45 percent of the global gross domestic product (GDP): China, India, the United States, Indonesia, and Brazil. "These are mostly new target populations," van Hylckama Vlieg said. Much of the research on the microbiome, diet, and health to date has focused on the "classical First World context," that is, Western Europe and the United States. Added to that is the trend in global aging. The age pyramid in 2015 is expected to be drastically different than it was in 2000, with large geographic differences in aging trends.

These demographic challenges are compounded by the fact that the food industry is often held responsible for the trend in obesity, although many other lifestyle factors contribute to obesity. Consequently, consumer associations are requesting more transparency on food composition, origin, and value for money. Yet there is an important role for the food industry to play in improving health for the global population. Science—in particular the science of the microbiome—is providing new tools and knowledge to manage these challenges. With respect to the microbiome, researchers are identifying a growing number of microbiota signatures and activities associated with health and disease (e.g., energy metabolism, production and availability of nutrients, cardiovascular health, cell proliferation and cancer, gut-brain axis and emotion, immune maturation and functioning, gut comfort, pathogen protection). More interestingly, in van Hylckama Vlieg's opinion, is that researchers are beginning to identify not just associations, but *causal* relationships between microbiome signatures and activities associated with specific health benefits. The food we eat is also the major source of growth for our gut microbiota and thereby may be an effective way to steer its composition and activity. The question is, How can we

[15] This section summarizes the presentation of Johan van Hylckama Vlieg.

leverage this science on the microbiome to develop products that maintain or improve health? Establishing causal relationship allows for microbes to be targeted, and the next step is to identify specific active ingredients and components that target these microbes and their impact on host health. Or, as van Hylckama Vlieg expressed, "Science provides increased rationale for functional food concepts using pre- and probiotics that bring a clear health benefit to consumers."

Leveraging the Microbiome for Health

In fact, humans have been leveraging the microbiome for a long time, initially through animal husbandry and consumption of fermented foods and moving toward science-based evidence for dietary intake. Fermented foods are important constituents of the human diet worldwide, and use of these foods dates back approximately 10,000 years (Evershed et al., 2008). These fermentations are often carried out with lactic acid bacteria. As van Hylckama Vlieg explained, lactic acid bacteria are also natural inhabitants of the human GI tract, so foods fermented by lactic acid bacteria are effectively supplementing the indigenous microbiota. In fact, lactic acid bacteria could be considered "domesticated" microbes. By substituting "microbe" for "animal" or "plant," they fit the *Webster's* dictionary definition of domesticated: "to adapt (an animal or plant) to life in intimate association with and to the advantage of humans."[16]

As an example of how Danone has leveraged the microbiome for health, van Hylckama Vlieg highlighted work on a fermented food product containing *Bifidobacterium animalis* subsp. *lactis* strain CNCM I-2494. The impact of FMP on the gut microbiome and host health was explored using a specific mouse model (*T-bet*$^{-/-}$ and *Rag2*$^{-/-}$ knockout mice, also known as TRUC mice) that spontaneously develops gut inflammation resembling human ulcerative colitis (Garrett et al., 2007). Garrett et al. (2007) reported that antibiotics reverse inflammation in TRUC mice, indicating a microbial etiology. Plus, when they co-housed TRUC and wild-type mice, they observed colitis in the wild-type mice as well, indicating the communicability of whatever gut microbes were associated with inflammation in the TRUC mice.

While a population of individuals with ulcerative colitis is far removed from Danone's target population, using that sort of extreme model can provide cleaner data and reveal mechanisms more readily than would be possible using other methods. Using the TRUC mouse model, Veiga et al. (2010) analyzed the TRUC gut microbiota by 16S rDNA sequencing and observed low levels of *Bifidobacterium* in the colon compared to non-inflamed (wild-

[16] See http://www.merriam-webster.com/dictionary/domesticate.

type) mice. They observed that feeding the TRUC mice 100 milligrams of FMP per day decreased intestinal inflammation (<0.0001 compared to TRUC mice fed the same amount of nonfermented food product) and increased levels of short-chain fatty acids (acetic acid, propionic acid, butyric acid). They also observed increased levels of lactate-consuming bacteria in the FMP-fed TRUC mice. The researchers showed "very elegantly," according to van Hylckama Vlieg, that the newly altered intestinal environment in FMP-fed mice inhibits growth of colitogenic bacteria. Research on the impact of FMPs on TRUC mouse microbiome and host health is ongoing.

Meanwhile, another study with the same FMP product on the gut microbiomes of healthy twin pairs and gnotobiotic mice demonstrated that FMPs do not cause any major perturbation of the dominant microbiota of healthy human subjects. However, this product does trigger distinct responses in the activity of the microbiome detected through transcriptomics (McNulty et al., 2011). Also, in both the mouse model and human samples, the metabolic activity of gut *Bifidobacterium animalis* subsp. *lactis* is dramatically altered. *B. animalis* subsp. *lactis* harvests and grows on the xylooligosaccharides derived from dietary components, which demonstrates that strain is becoming an active member in the gut microbial community.

Microbiome Biomarkers: Implications for Personalized Nutrition

In addition to research on mouse models and human samples, Danone is a partner of the MetaHIT Consortium. Acknowledging controversy about the biological relevance as expressed during the meeting regarding the existence of enterotypes, van Hylckama Vlieg remarked that regardless, "the observation is there" (Arumugam et al., 2011). The question is, What are the implications for the food industry? Do observations of enterotypes or any other clusters of markers in the microbiome indicate that people have specific nutritional needs, perhaps specific probiotic needs, depending on their microbiota compositions? It may be possible that these issues will lead to the emergence of personalized, or categorized, nutrition in coming years.

In preparation for these coming years, Danone is building and maintaining a culture collection of more than 3,000 microbes, mainly lactic acid bacteria. The collection includes strains isolated from multiple geographic areas (i.e., from various countries), ecosystems (i.e., from dairy products, cereals, plants, and stools), and time periods (i.e., from the 1960s through the present) and contains more than 80 species. They have sequenced nearly 100 genomes in the collection. "It is a very powerful platform for discovery," van Hylckama Vlieg said. Much of its power stems from its focus on strain diversity. In a comparative genome hybridization study of 42 strains of *Lactobacillus plantarum*, Siezen and colleagues (2010) found

that while about 70 percent of the genome of *L. plantarum* is shared among all strains, there are many genes not shared by all strains and many strain-specific genes.

Strain individuality raises the key question: What is the correlation with functional diversity? Currently, Danone investigators have been exploring the "pangenome" of multiple strains of two species, *L. rhamnosus* and *L. paracasei*, for gene-function correlations. Researchers are building a genome diversity database and coupling that with an extensive phenotyping program. Such studies will help to identify both common activities shared by many strains and specific features that can only be delivered by a few strains.

According to van Hylckama Vlieg, Danone scientists are looking at phenotypes related to carbohydrate utilization and short-chain fatty acid production, antimicrobial activity, and immune modulation. As an example of the type of results they are collecting, a sequencing analysis of 12 strains of *L. rhamnosus* and 30 strains of *L. paracasei* indicated that about 30 to 40 percent of the genome is "core," that is, shared among all strains. However, up to 25 percent of genes are strain-specific, with different strains having different immune function effects (i.e., based on an NF-kappa B-type screen on HT29 cells).

DISCUSSION

Most of the discussion during the question-and-answer period at the end of this session revolved around three major issues: (1) functional consequences of modulating the microbiome through food, (2) whether there are any known adverse effects of prebiotic and probiotic interventions, and (3) experimental design and study size.

Focus on Function

A recurring theme of the workshop was the importance of functional, not just compositional, changes to the microbiome as a result of dietary (or antibiotic) intervention. For example, as summarized in this chapter, Johan van Hylckama Vlieg mentioned research results demonstrating that Activia does not cause any major perturbation of the microbiota but does trigger distinct transcriptomic responses related to the metabolic activity of *Bifidobacterium animalis*. Also, George Fahey commented on the results of a study showing several positive metabolic outcomes associated with oligofructose consumption in mice. Finally, James Versalovic elaborated on the many ways that probiotics can impact host immunity. An audience member asked whether, given that food products appear to impact the microbiome not by recolonizing (so not by changing the taxonomic makeup of the mi-

crobiome), by rather through signaling (changing the way the microbiome is functioning), does it matter whether the food product in question is a live organism (i.e., a probiotic) or an inert substance (i.e., a prebiotic)? In other words, is the differentiation between probiotic and prebiotic a false dichotomy? Are the conceptual barriers between probiotic and prebiotic artificial?

Versalovic agreed that as the field moves forward, hopefully the dialogue will move beyond definitions and focus more on how food is impacting health via its effect on the microbiome. The value of the discovery of histamine as a microbially produced molecule that impacts host immunity isn't the histamine itself. Rather, its value is that "it has pointed us in a whole new direction—to look at amino acid conversions." Are other amino acids being converted by microbes? What other enzymatic machinery is the microbiome providing for the biochemical conversion of dietary information into biological signals? Versalovic said, "We're on entirely new trails in the wilderness." His research group is also studying another microbially catalyzed bioconversion of a host dietary substance into a biological signal, that is, glutamate into gamma-aminobutyric acid (GABA). He speculated on the potential regulatory role of the gut microbiome with respect to how much dietary information is actually being converted in vivo. "What's really intriguing here," he said, "is that that may be how the microbiome is really contributing to health and physiology … providing the enzymatic machinery, the metabolic pathways, at a particular location. It could be the GI tract. It could be the airways. It could be the oral cavity … providing that machinery that then allows [for the conversion of] the dietary content into [a] signal for the body."

Adverse Effects?

An audience member asked whether there were any known adverse effects of prebiotic or probiotic interventions. Mary Ellen Sanders replied that the National Institutes of Health (NIH) recently commissioned a review on probiotic safety that covered hundreds of prevention and treatment-of-disease studies of a variety of microbes (Hempel et al., 2011). The review concluded that most studies have not been adequately designed to address safety. Sanders speculated that the lack of safety data is likely due to the fact that probiotics have always been viewed as a food. They have not been viewed as drugs or as something that potentially could cause problems, at least not when administered to healthy people. So in the past, most researchers jumped right into efficacy studies, bypassing what would be the equivalent of a phase I drug study. Today, more researchers are designing their studies with safety factors in mind. Meanwhile, to Sanders's knowledge, at least for the most commonly researched microbes there is no or

very little association of use in a normal population with any adverse effects of real concern. There are some notable exceptions, she said. For example, Besselink et al. (2008) reported increased mortality after administration of probiotics to patients with severe pancreatitis. Sanders cautioned that not only do different microorganisms have different safety patterns, but that the health status of the host also influences safety.

Dan Levy emphasized the need to examine strain-specific safety effects. He said, "The tendency has been to say, well, we're exposed to all of these lactic acid bacteria with no adverse effects." But not all lactic acid bacteria are the same. While some strains may cause no adverse effects, that is not necessarily the case for all strains. This is especially worrisome when a strain is chosen for a unique property, that very uniqueness suggesting that the strain does not behave like others, particularly when selecting for strains intended to have specific and perhaps novel physiological effects on the consumer. "You have to stop lumping all lactic acid bacteria together," Levy said. "We're looking at specific physiological mechanisms associated with specific strains, and we have to study the good, the bad, and the ugly."

Experimental Design and Study Size

The discussion of microbiome markers of health and disease prompted a couple of comments on experimental design. Ellen Silbergeld expressed concern that too many studies on the microbiome are "almost entirely underpowered," raising serious questions about the value of the information being provided by those studies. If a study is too underpowered to provide any evidence of an effect, then what is the value of that study? "If you really want to move this field forward," she said, "you really have to start considering your study design." In response, Johan van Hylckama Vlieg remarked that many small studies, such as the twin study that he mentioned, are intended to be exploratory and hypothesis-generating. They are not intended to yield the same type of results that large-scale clinical trials provide. Silbergeld then wondered whether any of the larger studies actually meet the criteria of a clinical trial. It is not clear that any do. Versalovic questioned whether large-scale clinical trials are even appropriate for microbiome studies. Given such extreme individual-level variation in microbiome composition and function, he said, "I don't know that we can do trials the same way anymore," indicating the need for further discussion on issues of study design.

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6

Societal and Policy Implications

A probiotic is only as effective as its ability to remain viable until it reaches a place in the human gastrointestinal (GI) tract where it can exert its effects, David Julian McClements explained (see a summary of his presentation in Chapter 5). If the viability of a probiotic is lost during storage within a food product or in the human body en route to its destination, then the beneficial health effects may not be realized. The challenge doesn't end there. In the final speaker session of the workshop, participants addressed other, nonbiological challenges—consumer, regulatory, and industry challenges—to realizing the potential of food as a primary modulator of the microbiome for health. This chapter summarizes that discussion.

HOW AMERICANS EAT AND DRINK TO IMPROVE HEALTH[1]

Research conducted by the NPD Group shows that consumer behavior is stable over time. The NPD Group collects data both on usage, that is, food preparation and consumption (e.g., What did you eat? What were the ingredients? Did the product have any kind of health claim?) and on the planning and acquisition of food (e.g., What did you have on hand in your pantry? Why was a certain item on your grocery list?). Darren Seifer noted that even during difficult economic times when people are watching their budgets, consumer behavior remains mostly stable. For example, NPD data indicate that the top breakfasts consumed in the United States, based on annual eatings per capita, were more or less the same in 2011 as

[1] This section summarizes the presentation of Darren Seifer.

in 2001. In 2001, the top five breakfasts were, in order, cold cereal; eggs or omelets; bread; hot cereal; and pancakes, waffles, or French toast. In 2011, the top five breakfasts were, again in order, cold cereal; eggs or omelets; hot cereal; pancakes, waffles, or French toast; and bread. According to Seifer, pancakes, waffles, and French toast have moved up to the number four spot because those products are being delivered in more easy-to-prepare fashions, such as instant or frozen.

The same stability has been observed for lunch and dinner. NPD data indicate that the top five lunches consumed in the United States in 2001, again based on annual eatings per capita, were, in order, sandwiches (including burgers), soup, poultry, pizza, and salads. In 2011, the list and order were the same. The top five dinners consumed in the United States in 2001 were poultry, sandwiches (including burgers), beef, Italian dishes, and homemade or mix "variety." In 2011, preferences shifted, but only slightly, to sandwiches (including burgers) as the top dinner, followed by poultry, beef, Italian dinners, and pizza. Seifer explained that as with breakfast pancakes, waffles, and French toast, part of the reason for pizza moving up into the top five list is that it is being sold in an easy-to-prepare frozen form.

The slight shifts in consumer behavior around frozen foods suggest that underlying consumer behaviors can change, albeit slowly. Changes take years, if not decades. If one were to examine just the past several years, NPD data do not show much change in the percentage of meals with a main dish coming in frozen form. In looking back to the mid-1980s, however, there has been a noticeable increase in frozen items being served at all three main meals. For breakfast, 4 percent of main dishes served in 1984 were purchased in frozen form. That figure doubled, to 8 percent, by 2011. For lunch, 7 percent of main dishes served in 1984 were purchased in frozen form. That figure also doubled, to 14 percent in 2011. For dinner, 15 percent of main dishes served in 1984 were frozen. By 2011, that figure had increased to 23 percent.

The growing preference for foods that do not need to be cooked is corroborated with other NPD data on the top 10 fastest-growing in-home foods and beverages. Based on point change in percentage of Americans consuming a product at least once in a 2-week period between 2001 and 2011, the top 10 fastest-growing in-home foods and beverages were yogurt (15), bars (10), chips (10), bottled water (10), nuts and seeds (9), pizza (8), fresh fruit (7), poultry sandwich (7), specialty Italian (7), and cheese (6). Seifer noted that fresh fruit is on the list because of changing demographics; it is popular among older adults as well as among children. A common characteristic of these foods is that they require very little, if any, preparation.

Yogurt

Yogurt consumption has more than doubled over the past decade, from 13 annual eatings per capita in 2001 to 30 in 2011. "Certainly it is something to keep watching and monitoring," Seifer said. However, compared to other snack categories, it is still small. People eat salty items three times more often than they do yogurt, and even though people drink less milk than they used to, they still consume more than four times as much milk as yogurt.

The fast growth of yogurt consumption raises the question, Why are people choosing yogurt? NPD data show that the number one reason people choose yogurt is because it is nutritious and has a health benefit (15 percent). This is different from most food items, Seifer noted. Usually taste is the top reason for choosing a food. In the case of yogurt, however, taste is the second reason (13 percent), followed by "was hungry" (10 percent), "healthy start to day" (8 percent), "favorite snack" (6 percent), "simple and easy to eat" (6 percent), "routine or habit" (5 percent), "better than other choices" (5 percent), "hold me over until next meal" (4 percent), and "low in fat or calories" (3 percent).

The Nutrition Facts Panel: What Are Consumers Looking For?

When people look at a Nutrition Facts Panel, what are they looking for? NPD data indicate that consumers are looking mostly for total calories (49 percent), followed by sugars (43 percent), total fat (43 percent), sodium (41 percent), calories from fat (34 percent), total carbohydrates (33 percent), serving size (33 percent), saturated fat (33 percent), servings per container (28 percent), cholesterol (28 percent), and dietary fiber (25 percent). Seifer remarked that even though calories are the number one thing that people look for, studies indicate that only about 10 percent of people know how many calories they should be consuming on a daily basis.

More people are looking for sugar content than in the past (43 percent in 2010 compared to 39 percent in 2004). Seifer characterized the growing interest in sugar as a remnant of the "Atkins and low-carb craze" and a function of the aging population with more health concerns to manage, including diabetes. People are also looking for fiber content more often than in the past (25 percent in 2010 compared to 22 percent in 2004), a trend that Seifer suggested may also be related to our aging population and the fact that dietary fiber is something that older adults are concerned about. Another trend is the growing interest in sodium (41 percent in 2010 compared to 34 percent in 2004), again probably because of changing demographics and greater concern about sodium among older adults. Again, as with calories, even though people are expressing more interest

in sodium content, they are not necessarily consuming sodium within the recommended levels. In fact, NPD Group data indicate that all age groups are consuming too much sodium. Among adults, seniors (65 years and older) consume the least amount of sodium, at an average 2,912 milligrams per day. The maximum recommended daily intake level, however, is 2,300 milligrams for the general population (the recommended average daily sodium intake level is 1,500 milligrams or less, particularly for older adults; African Americans; and anyone with hypertension, diabetes, or chronic kidney disease).

American consumers are not looking at fat content as often as in the past (43 percent in 2010 compared to 48 percent in 2004), according to NPD data. At one time, checking fat content was second only to checking calories. Nor are they looking at cholesterol as much as they did in the past (28 percent in 2010 compared to 32 percent in 2004). Like fats, cholesterol used to be a primary health concern (in the late 1980s). Calcium content is being checked less often as well (9 percent in 2010 compared to 10 percent in 2004).

Seifer emphasized that not only are people looking for different things on the Nutrition Facts Panel, they are also looking for ways to enhance, not restrict, their diets, according to NPD data. It is easier to add ingredients to one's diet than remove them. This may partly explain yogurt's increasing popularity, according to Seifer. The simple act of eating yogurt provides health benefits. Consumers would rather ingest their way to health than restrict their way to health. In 2010, more people were trying to add whole grains, dietary fiber, and antioxidants to their diets than in 2004. Also in 2010, not nearly as many people were as cautious about fat, salt, cholesterol, sugar, and caffeine as have been in the past. Along the same line, dieting is also on the decline, with a smaller percentage of people reporting being on any diet in 2011 compared to 2002. Older adults report being on a diet more often than any other age group.

Consumer Behavior Around Probiotics and Prebiotics

When probiotics were first introduced into the marketplace, consumers were confused. According to data collected by the NPD Group, in 2006 more adults were trying to cut down on or avoid probiotics (13 percent) than get more probiotics into their diets (10 percent). The trend has shifted, with more adults trying to get probiotics into their diets (24 percent) than avoid them (10 percent) in 2010. Prebiotics are still a challenge, with only 15 percent of adults trying to get more prebiotics in their diets in 2010 and 12 percent trying to cut down or avoid prebiotics.

Seifer emphasized that consumers tend to be reactive, not proactive. That is, they tend to react when there is a need, such as a medical condi-

tion (e.g., diabetes, heart disease, high cholesterol, high blood pressure). For example, many people with diabetes manage their sugar intake by eating foods that either lack sugar or are higher in whole grains. Compared to the total population, they eat more bread, eggs, soup, hot cereal, crackers, and seafood, and they eat fewer cookies, Italian dishes, pizza, "mac and cheese," bars, toaster pastries, and brownies. Seifer noted that seniors (65 years and older) and older baby boomers (55-64) are more likely to experience medical conditions that drive the consumption of fruits, vegetables, and other foods perceived as nonharmful.

Expanding the user base of probiotics and prebiotics beyond those who have a need for additional microbes will require a marketing effort. Seifer referred to the "four P's" of marketing: product, price, promotion, and place. In addition to alleviating some of the confusion that still surrounds use of the words "probiotic" and "prebiotic," consumers may have to be educated on the health benefits of adding more microbes to their diet. "Be patient," Seifer advised. "It is going to take time for this to catch on."

CONSUMER INSIGHTS FROM THE INDUSTRY PERSPECTIVE[2]

The probiotic market is one of the fastest-growing sectors in the functional food market, according to Peggy Steele. In 2001, probiotics accounted for $25 billion in sales worldwide. The sector is expected to continue to grow at more than 6 percent annually, yielding an estimated $32 billion in annual sales by 2015. Regionally, North America, Latin America, Eastern Europe, and Asia Pacific are expected to see the strongest growth in coming years. Growth in the already mature markets of Western Europe and Japan will be slower.

Yogurts account for the majority of new products being launched as probiotics (about 75 percent), with the remainder including baby food and baby milk powder, drinks, cheese, other dairy (milk, cream, kefir), frozen desserts, and other products. The current U.S. market for probiotic yogurt is more than $1 billion, representing about one-quarter of the overall refrigerated yogurt segment. Over the past several years, the probiotic yogurt market has grown more quickly (10 percent) than the market for nonprobiotic yogurts (6.5 percent). "Yogurt is really the dominant delivery vehicle for probiotics right now," Steele said. She referred to Darren Seifer's remarks on the appeal of the health benefits of yogurt to consumers.

Yet as markets for probiotic yogurt and other products continue to grow, Steele observed a trend toward stricter regulation and enforcement of health claims on those products. The European Food Safety Authority (EFSA), the U.S. Food and Drug Administration (FDA), Health Canada,

[2] This section summarizes the presentation of Peggy Steele.

and other regulatory agencies have taken considerable action to control and regulate probiotic health claims. In Steele's opinion, the level of science being required to support certain claims is approaching "pharma" level documentation. She expressed concern that not all manufacturers will have the funding or resources needed to provide such support, resulting in consolidation and even elimination of some competitors. While probiotics that successfully pass regulatory scrutiny will likely instill consumer confidence and acceptance of probiotic products and thereby increase their market share, those that fail regulatory scrutiny could have the opposite effect.

Because of the changing regulatory pressures, major players such as Danone are softening their claim language. Previously, Danone's claims on its probiotic products were focused on structure-function claims. Now the claims are focused on nutrition or general function, and some products make no claims at all. Interestingly, Steele noted, despite the increasing regulatory pressure and changes in claim language, the probiotic market continues to show remarkable growth. Whether that growth is sustainable is difficult to predict.

In addition to regulatory restraints, other key market constraints include the presence of substitutes (e.g., other functional ingredients for gut and immune health such as vitamin C); lack of awareness of the word "probiotic," especially in the United States; and challenges with product stability (i.e., probiotics are sensitive to many food and beverage applications). Key market drivers include growing benefit substantiation (i.e., clinical documentation on an expanding list of conditions); health care provider endorsements; increasing awareness as a result of companies such as Danone and Yakult promoting digestive health; a trend toward self-care; and the perceived immediate impact of probiotic products (e.g., feeling the benefit of taking Activia after only 2 weeks of regular consumption).

Positioning of Probiotics: Types of Claims

In the United States, there are three general categories of probiotic claims: (1) content claims, (2) structure-function claims, and (3) health claims. Examples of content claims are "contains *L[actobacillus] acidophilus* bifidobacteria," "contains live and active bacteria," and "probiotic." These require the least amount of documentation. Examples of structure-function claims are "supports good digestion," "promotes a healthy digestive and immune function," and "helps naturally regulate your digestive system." Structure-function claims require more documentation than content claims and, according to Steele, resonate very well with consumers. Examples of health claims are "reduces the risk of cancer," "reduces IBS [irritable bowel syndrome]," and "reduces incidence and severity of chronic constipation." These require the greatest amount of documentation. Probi-

otic health claims cannot make any reference to treatment or mitigation of disease or disease symptoms. If so, they are considered drug claims, which require pharmaceutical approval.

The most common probiotic product claims are digestive and immune structure-function claims. In the United States, 28 percent of all probiotic yogurts make structure-function claims. About one-third of those claims are for digestive health only and another one-third for both digestive and immune health. Only 3 percent are for immune health only.

U.S. consumers are becoming increasingly aware of the digestive health benefits of probiotics. In an International Food Information Council (IFIC) online survey, when asked about awareness of the digestive health benefits of probiotics, the percentage of respondents answering positively increased from 58 percent in 2007 to 81 percent in 2011.[3] Steele wondered whether a similar awareness among European Union (EU) consumers might be driving growing sales in probiotic yogurt despite the softening of claim language.

Steele urged manufacturers to focus on clear, relevant, and substantiated claims on the front of their products' packages. A DuPont survey conducted in 2011 showed that 75 percent of consumers look at the front of the package for nutrition information. Most people tend to read the Nutrition Facts Panel and ingredient lists only when they are making a change.

What Can Industry Do?

Steele identified three major activities of industry that can move the field of probiotics forward in the face of a changing regulatory landscape: (1) demonstrate efficacy and help the scientific and regulatory communities to recognize the effects of probiotics on human health; (2) educate and increase dialogue with doctors, nutritionists, key opinion leaders, and journalists to communicate the results of human studies conducted on probiotics; and (3) explore new end points (i.e., different populations, new health areas).

As an example of efficacy research in which Danisco and DuPont[4] have been involved, Steele highlighted a study on two probiotics (*Lactobacillus acidophilus* NCFM and a blend of *L. acidophilus* NCFM and *Bifidobacterium lactis* Bi-07) in children aged 3-5 years (Leyer et al., 2009). The study was a 26-week prospective, double-blinded trial of 326 children randomized across three treatments (the two probiotic treatments and one placebo

[3] More information on the IFIC 2011 Functional Foods/Foods for Health Consumer Trending Survey is available online at http://www.foodinsight.org/Resources/Detail.aspx?topic=2011_Functional_Foods_Foods_For_Health_Consumer_Trending_Survey (accessed August 20, 2012).

[4] In May 2011, Danisco was acquired by DuPont.

control). The children were administered 5×10^9 colony-forming units (CFUs) twice a day. The study was conducted during the cold and flu season (i.e., from November to May). Researchers observed a significant reduction in respiratory tract infections in both treatment groups. Also, children who received the blended probiotic demonstrated an 82 percent reduction in antibiotic use and a 46 percent reduction in number of sick days. The findings were corroborated by a more recent (manuscripts in preparation) efficacy study of 474 adult athletes showing that the same blended probiotic led to a 33 percent reduction in respiratory infections and a 35 percent reduction in medication use over a 150-day period during the cold and flu season.

As an example of how DuPont[5] has contributed to education, Steele noted the national media coverage of the company's HOWARU product launch. She also commented on Danone's pioneering work in educating consumers about probiotic yogurt and the company's role in promoting digestive health, including its use of a celebrity (Jamie Lee Curtis) to promote the digestive benefits of probiotic yogurt.

With respect to exploring new end points, Steele identified the need to address different age groups and adapt probiotic formulations accordingly. For example, older adults might require different probiotic blends than younger adults or infants and children. Reiterating some of what Julian McClements covered during his presentation, Steele explained that a probiotic must remain viable and in sufficient quantity until it actually delivers its benefit. A whole host of food factors could impact viability, including formulation, processing, storage or distribution, and shelf life. As just one example, she referred to data on the survival of an *L. casei* strain in orange juice. Over the course of its 60-day shelf life, the probiotic population decreased in size about 10-fold. DuPont scientists are also testing different food matrices both in simulated GI tract conditions and in the clinic to see which matrices are most protective of a probiotic as food is digested (Ibrahim et al., 2010; Makelainen et al., 2009).

Finally, DuPont scientists are investigating new health end points. For example, Amar and colleagues (2011) reported that *B. lactis* 420 can reverse high-fat diet-induced diabetes in mice. In addition to impacting blood glucose, insulin secretion, and insulin sensitivity, the probiotic also reduced fat mass. The researchers linked the effects to reduced plasma lipopolysaccharide (LPS) levels and reduced tissue inflammation. As another example, Shu et al. (2000) reported significantly improved survival rates among mice that were administered Danisco's HOWARU Bifido (*B. lactis* HN019) and then challenged with a single dose of *Salmonella typhimurium*. The results have implications for pathogen-induced diarrheal disease in humans.

[5] The HOWARU product line was launched by Danisco.

PROBIOTIC AND PREBIOTIC HEALTH CLAIMS IN EUROPE: SCIENTIFIC ASSESSMENT AND REQUIREMENTS[6]

EFSA has responsibility across all EU countries for regulating nutrition and health claims on foods including probiotic and prebiotic products. EFSA is not a decision maker, Seppo Salminen explained. Rather, it is an independent risk assessment and scientific advice authority. The role of EFSA in the scientific substantiation of nutrition and health claims is based on a 2006 European Parliament regulation.[7] Prior to the 2006 regulations, claims were regulated through directives, with each member state acting independently.

While the new EU claim regulation addresses free and fair trade of goods, including foods, and promotion of innovation, its most important principle is consumer protection: "Nutrition and health claims on food must be substantiated by scientific evidence." The regulation creates several new challenges for claim applicants. First, evidence should be collected from a population that is representative of the generally healthy population or from which results can be extrapolated to the general population, which Salminen noted varies even within Europe (e.g., there are significant differences in microbiota composition and activity in individuals from northern versus southern Europe). Second, the evidence must be considered "generally accepted science," which Salminen noted is a difficult criterion for microbiota research. Finally, scientific assessment of proposed claims must address causality. Salminen referred to the comments of many previous speakers and workshop participants on the issue of causality (i.e., that most of the evidence on diet-microbiome-health relationships is associational, not causal).

Because of these challenges, EFSA issued a guidance document to assist applicants in preparing and presenting their applications for authorization (EFSA, 2011a). The guidance document addresses both format and criteria for substantiation. Salminen noted that it is constantly being reviewed and updated and that EFSA also conducts stakeholder consultations. EFSA also published an additional and more specific guidance document on the scientific requirements for claims on gut health and immunity (EFSA, 2011b). The document summarizes experience from earlier assessments, gives guidance on factors associated with gut health and immunity, summarizes beneficial effects or beneficial implications that have already been considered, attempts to explain why some studies are applicable and others are not, and provides some outcome measures. The guidance is not exhaustive, Salminen warned. That would be impossible. Evaluations are conducted on a case-

[6] This section summarizes the presentation of Seppo Salminen.

[7] *Regulation on Nutrition and Health Claims Made on Foods*, EC No. 1924/2006 of the European Parliament and of the Council (December 20, 2006).

by-case basis. Furthermore, the guidance is a living document and will be updated as appropriate.

Types of Nutrition and Health Claims That Can Be Made About Probiotic and Prebiotic Food Products

As in the United States, three types of nutrition and health claims are allowed in the European Union: (1) nutrition claims based on scientific assessment of a benefit, (2) function claims about the maintenance or improvement of a function, and (3) disease risk reduction claims about the reduction of a risk factor for a human disease and claims concerning children. With respect to disease risk reduction claims, disease risk reduction must be demonstrated using a commonly accepted risk factor and changes in relevant biomarkers that relate to the risk of the particular disease. These categories of claims were not decided by EFSA, but rather by "word of law."

Examples of function claims are claims about bowel function and constipation, gastrointestinal discomfort, and defense against pathogens. In order to make a function claim about bowel function and constipation, for example, one would define an average healthy consumer and then show that the probiotic or prebiotic in question can reduce or somehow improve bowel function. Salminen commented on the availability of validated questionnaires for use in assessing GI discomfort and the ways to gather these data in a scientifically acceptable manner. Making a function claim about pathogen defense requires demonstrating a reduction in numbers of specific pathogens (i.e., "real" pathogens associated with the particular risk, not opportunistic pathogens). An example of a disease risk reduction claim is a claim about LDL (low-density lipoprotein) cholesterol as a recognized risk factor for heart disease. As required by the regulation, the risk factors must be physiologically relevant. Salminen emphasized that there is no exhaustive list of recognized risk factors, reiterating that EFSA conducts assessments on a case-by-case basis.

Characterizing and Evaluating Probiotic and Prebiotic Products in the European Union

There is no legal EU definition of either "probiotic" or "prebiotic." According to the Food and Agriculture Organization-World Health Organization (FAO-WHO, 2002) definition, probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host." A prebiotic was defined by FAO as "a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota" (Pineiro et al., 2008). On the basis of these definitions and other parameters laid out by FAO and WHO, Salminen explained

that in order for something to be considered a probiotic (or prebiotic), its health effects and safety must be demonstrated, with an emphasis on data from human studies, and strains must be clearly identified and deposited in public culture collections. Because of the uniqueness of each probiotic (and prebiotic) strain, the health effects for each individual strain or strain combination should be documented separately, according to Salminen.

Yet a number of bacteria currently being marketed in the European Union as probiotics have no demonstrated health-promoting properties, and different strain combinations are advertised without any proven association with health benefits. One of the goals of the 2006 health claim regulation was to improve consumer protection by more clearly identifying actual probiotic and prebiotic products and their benefits to the consumer.

As part of its task, EFSA was required to assess existing health claims in each individual EU member state. These assessments required identifying and characterizing the probiotics in use; evaluating relevant studies, with an emphasis on controlled human intervention studies; and assessing whether the proposed health relationship is something that consumers can understand.

Characterization alone has been a challenge. The purpose of characterization is to assure EFSA that the substance for which a claim is made is the same as that for which the evidence on efficacy is provided. Until a substance is characterized, EFSA cannot conduct a health claim assessment. Deposit to an international culture collection is key. The strain does not have to be publicly available, Salminen noted, but it should be available to regulatory officials. In its assessment of existing health claims, EFSA has identified more than 100 probiotic products that could not be characterized because of a lack of data on the strain used.

The most important component of a health claim assessment is human intervention studies (van Loveren et al., 2012). For disease risk reduction claims, the manufacturer needs to show that the product causes change in a generally accepted risk factor in a normal, healthy population, Salminen noted. It is also important for human intervention studies to be supported by animal model or other mechanistic studies.

Salminen acknowledged the challenge of demonstrating a change in a normal, healthy population. To illustrate the difficulty, he mentioned a study on a milk-based drink containing a combination of *L. rhamnosus* CG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *shermannii* JS, and a *Bifidobacterium* strain. The goal was to see if a daily dose of 4×10^9 CFU, with equal amounts of each bacterial strain, would reduce GI discomfort in a normal, healthy population of individuals. According to Salminen, although two "quite nice" clinical studies were conducted (both randomized, placebo-controlled, double-blind intervention studies), they were conducted using two different strains of *Bifidobacterium* (EFSA,

2008). One mixture had *B. animalis* ssp. *lactis* Bb12 (DSM 15954), the other *B. breve* 99 (DSM 13692). Because the two studies used the same product but in two different probiotic combinations, the combined evidence was insufficient to establish a causal relationship between the product and a reduction in abdominal discomfort. Salminen noted that it is not clear whether the difference between each strain would have an impact on effect, but noted the fact that there are biological differences among different strain combinations.

The health effect of xylitol, a prebiotic, on caries risk is an example of a well-supported health claim. Dental caries is very common among Europeans, and a review article estimated the prevalence of caries to be up to 100 percent, depending on the country (Bagramian et al., 2009). A well-recognized risk factor for dental caries is thickness of dental plaque at specific sites in the mouth. Several intervention studies have demonstrated a reduction in dental caries risk as a result of chewing xylitol-sweetened gum, as reviewed in Deshpande and Jadad (2008).

In conclusion, Salminen remarked that most researchers do not usually think about health claims when designing their studies, but in many cases, redirecting studies to address health claims requires making only small changes to the study design.

EVALUATION OF VIABLE MICROBES USING REGULATORY REQUIREMENTS DEVELOPED FOR NONVIABLE INGREDIENTS[8]

FDA is, as Dan Levy expressed, "a creature of the statutes." FDA must operate within parameters laid out in the law, which, in the case of foods, is primarily the Federal Food, Drug, and Cosmetic Act (FD&C Act) initially passed in 1938[9] and amended many times thereafter. Of particular relevance to probiotic-containing food products is the 1994 Dietary Supplement Health and Education Act (DSHEA).[10] The DSHEA defined a dietary supplement as "a product (other than tobacco) intended to supplement the diet that bears or contains one or more … *dietary ingredients*." The statute includes a list of substances that can be dietary ingredients. Substances not on the list cannot be dietary ingredients. The list consists of vitamins, minerals, amino acids; herbs or other botanicals; dietary substances for use by man to supplement the diet by increasing total dietary intake; and concentrates, metabolites, constituents, extracts, or combinations of any of the

[8] This section summarizes the presentation of Dan Levy.

[9] For more detailed information on the FD&C Act, visit the FDA website: http://www.fda.gov/regulatoryinformation/legislation/federalfooddrugandcosmeticactfdcact/default.htm.

[10] Dietary Supplement Health and Education Act of 1994, Public Law 103-417, 103rd Cong. (Oct. 25, 1994).

above ingredients. The only appropriate category for probiotics is "dietary substance for use by man to supplement the diet by increasing the total dietary intake." The draft guidance suggests that this definition is limited to substances previously in the diet, suggesting that an entirely new bacterium must enter the food supply as a food other than a dietary supplement.

Pre-Market Notification for New Dietary Ingredients: Implications for Probiotics

The FD&C Act requires pre-market notification to FDA describing the safety of a dietary supplement containing "new dietary ingredients." New dietary ingredients are defined as dietary ingredients not marketed in the United States prior to October 15, 1994. However, there is an exemption from the notification requirement for a dietary supplement containing only dietary ingredients that have been present in the food supply "as an *article used for food* in a form in which the food has not been *chemically altered*." When it passed the DSHEA, Congress included a "legislative agreement" describing what "chemically altered" does *not* mean. It does not include minor loss of volatile components, dehydration, lyophilization, milling, tincture or solution in water, slurry, powder, or solid in suspension. Yet even with this list, there are uncertainties. For example, what does "tincture or solution in water" mean? Levy commented that everyone would probably agree that making an extract by soaking leaves in hot water would not be "chemically altered." More complicated extractions, such as the use of organic solvents and chromatography, make more significant changes in the chemical composition of the extract, changes that may be described as chemically altered.

The issue of whether or not something is chemically altered is especially challenging for live microbial organisms used in food production because chemical characterization is usually not used to describe the difference between microorganisms. For example, when a strain of a *Lactobacillus* species that has been used to ferment cheese is selected to produce a variant having properties that optimize its use in an industrial fermentation for the production of a dietary supplement, is the selected strain chemically altered relative to the strain that was in the original dairy product?

The FDA Food Safety Modernization Act (FSMA) of 2011[11] required FDA to publish guidance on new dietary ingredients to help industry and other stakeholders understand when new dietary ingredients (NDIs) require pre-market notifications and what those notifications should include (see Box 6-1). FDA published a draft guidance in July 2011, with a comment

[11] FDA Food Safety Modernization Act of 2011, Public Law 111-353, 111th Cong. (Jan. 4, 2011).

BOX 6-1
FDA Draft Guidance on New Dietary Ingredients: Implications for Probiotics

FDA published draft guidance in July 2011 to help industry and other stakeholders understand when new dietary ingredients require pre-market notification and what those notifications should include. With respect to live microbial ingredients, here is what the guidance recommends for identity and safety. The italicized text is text that Levy highlighted during his presentation.

On identity, the guidance reads: "FDA considers each strain of a bacterial or yeast species to be a separate ingredient. You should explain how your strain was obtained and how it varies from other members of the same species. If your strain was genetically modified using either random mutagenesis or bioengineering, you should describe the process used and how you characterized the properties of the new strain.… You should include a complete description of the organism, including: the strain, methods used to establish the identity of the strain, such as identification by internationally recognized third-party repositories (e.g., the American Type Culture Collection), and the relationship of the strain to the strain(s) of the same species used to establish the history of use or other evidence of safety for the dietary ingredient.… For organisms that come from species or genera with pathogenic strains: Is there a consensus that there are valid scientific ways to distinguish between pathogenic and non-pathogenic members or to prevent horizontal transfer of genes for pathogenic traits?"

On safety, the guidance reads: "You should identify any human *pathogens that are phylogenetically related* to the microbial NDI at the species or genus level.

deadline of December 2011 (FDA, 2011b). At the time of the Institute of Medicine (IOM) workshop, FDA was still reviewing the more than 100,000 pages of comments received. The need for the guidance was illustrated by FDA's objections to many NDI notifications, many of which were based on uncertainty about the identity of the ingredient and comparison of that ingredient to the ingredient(s) used in the safety comparison. Levy noted that the challenge is very similar to what EFSA is dealing with in terms of characterization of strains in probiotic products (see the summary of Seppo Salminen's presentation). Many of the FDA objection letters say something like, "Without such information, it is unclear how the product you intend to market is qualitatively and quantitatively similar to the substances described in the information that you rely on as evidence of safety or how that information forms the basis for a reasonable expectation of safety under the intended conditions of use." Levy reminded the workshop audience that guidance "does not bind anybody to do anything." Rather, "it is just our best thinking" at the time it is published.

FDA is addressing the challenge of whether or not a live microbial

You should *identify any toxins*, classes of toxins, *or other deleterious substances* known to be present in the same species or in a phylogenetically related family or genus. You should also document the absence (or the amount, if present) of such toxins or other deleterious substances in the NDI. You should *document resistance to any clinically relevant antibiotics*, and if applicable, the genetic nature of the resistance. If the microbial NDI is resistant to any clinically relevant antibiotics, it is also recommended that you perform an assessment of the ability of the antibiotic resistance genes to mobilize and transfer to human pathogens under the conditions of use of the dietary supplement.… If your notification cites the history of use of a live microorganism as evidence of safety, FDA recommends a *careful assessment of the relative level of historical exposure* compared to the proposed conditions of use of the NDI, including a discussion of how the form of the dietary supplement and any excipients used in it affect delivery of the NDI to various points in the human gastrointestinal tract.… If history of use data are inadequate to support the safety of the microbial NDI, you should include safety studies in *humans or appropriate animal models* in your notification. FDA considers pigs to be the most appropriate animal model for the human digestive tract. Human or animal safety studies should include measurements of the persistence of the organism in the body after administration, the ability of the organism to translocate outside of the gastrointestinal tract, and tolerance of the ingredient using the proposed serving form. *Because this is a rapidly evolving scientific discipline, FDA recommends that notifiers be familiar with the state of the recent scientific literature at the time the notification is submitted.*"

SOURCE: FDA, 2011b.

ingredient has been chemically altered by applying the logic used for botanical extracts. For botanical extract composition, the draft guidance recommends supplying information on solvent used, extraction ratios, and special cultivars or harvest conditions. The new guidance document contains specific examples of specification sheets for a botanical extract dietary ingredient. The draft also suggests that detailed information on the analytic methods used to characterize the composition of the new dietary ingredient be included.

The guidance document also includes recommendations for describing a live microbial ingredient. Although FDA does not require the use of scientific names for microbes, the guidance encourages it. Because the guidance suggests that each strain of a bacterial or yeast species be a separate ingredient, the guidance encourages applicants to explain how their strain was obtained and how it varies from other members of the same species. Levy recognized that the guidance on identity for live microbial ingredients is not detailed. He explained that the lack of scientific consensus on strain specificity, including how to differentiate between strains, makes it

difficult for FDA to be clearer on how to demonstrate strain specificity. As an illustration, he described the difficulty microbiologists have in clearly differentiating pathogenic versus nonpathogenic strains of the same species, such as *Escherichia coli*.

Just as there is no scientific consensus on how to distinguish among strains, there is no scientific consensus on how best to evaluate the safety of a microbial culture. This is unlike nonmicrobial ingredients, for which the guidance provides references to standard toxicology assays. "There are protocols out there that tell one exactly how to do each of those studies," Levy said. Not so for live microbes. As with identity, the guidance provides only general advice about what information should be submitted to demonstrate safety, since this is a rapidly changing area of science.

Research on the microbiome is such a rapidly advancing field that it is premature for FDA to develop specific recommendations at this time, Levy remarked. He referred to the same Agency for Healthcare Research and Quality (AHRQ) study on probiotic safety that Mary Ellen Sanders had mentioned previously (Hempel et al., 2011). The authors of that study concluded that while there is no evidence that probiotics are unsafe, they did not have a great deal of confidence in their conclusion. The review found that most trials reported in the scientific literature are either poorly designed or poorly reported, making it difficult to evaluate safety. Many do not mention which strain was used or how the strain was prepared. Others do not explain how adverse events were monitored. The review also found that there has been no real effort to examine long-term safety risks (either safety of long-term exposure or long-term effects that show up after the conclusion of a trial). "Nobody is studying long-term safety in a systematic way," Levy said.

Because the guidance document cannot be specific about areas in which science is rapidly developing, Levy encouraged companies to engage in dialogue with FDA before submitting NDI notifications. "We are using these organisms in new way," he said. "[The] organisms … sound familiar because they have been present in fermented foods for a long time, but we are selecting them to have properties that were not really selected for previously. That is an intended use that is new and requires a dramatic new efficacy and safety evaluation paradigm."

Questions

Levy's presentation prompted several questions on evaluating probiotic safety. A workshop audience member asked if FDA's "typical approach" of using an existing benchmark and examining marginal changes from that benchmark could be applicable in the case of live organisms. For example, if an organism already exists in a yogurt product, would it be acceptable

to FDA to examine the differences between the organism as it appears in that yogurt product and the organism as it appears in a supplement? Levy responded that the answer lies in intended use or effect. If the intended effect of the organism in the original product was the same, then yes, that is a valid approach.

There was a question about a hypothetical probiotic product designed to be administered to cesarean section (C-section) babies in an effort to establish a microbiota that is similar to that developed by a vaginally delivered baby. Would such a product be considered by FDA to be a food or a drug? Levy replied that it is unclear what that type of product would be considered. He would encourage discussion with "representatives from both sides of the agency."

Another participant asked under what circumstances a probiotic is considered a new dietary ingredient as opposed to a food additive. Levy described that type of categorization as "a gray area" because it often depends on how the food is intended to be marketed. He also pointed out that each category has its own safety evaluation paradigm. "However it is represented," he said, "you have to meet the requirements of that particular paradigm."

Finally, when asked whether any probiotics or prebiotics are being self-determined as generally recognized as safe (GRAS) food ingredients, Levy replied, "It would not surprise me if a lot of people are doing GRAS self-determinations." However, because GRAS self-determination does not require FDA notification, FDA does not have a complete list of such determinations although there are procedures that allow voluntary notification of FDA. GRAS determinations, however, are limited to ingredients in conventional foods, not dietary supplements. Mary Ellen Sanders added that she knew of about six GRAS notices for probiotics posted on the FDA website.

HEALTH CLAIMS AND FALSE ADVERTISING[12]

The U.S. regulatory landscape for foods is governed not just by FDA, but also by the Federal Trade Commission (FTC). Michelle Rusk explained how the two agencies share authority on marketing claims not just for foods, but also for supplements, drugs, and other health and medical products. Specifically, through a long-standing liaison agreement, FDA has primary authority for claims appearing on labeling or product packaging, while FTC has primary authority for claims appearing in advertising (with the exception of prescription drugs, over which FDA has authority on both labeling or packaging and advertising). By "advertising," Rusk was refer-

[12] This section summarizes the presentation of Michelle Rusk.

ring to broad-sense marketing, not just television and print advertising but anything intended to promote a product.

Despite their coordination and shared authority, the legal frameworks of FDA and FTC differ in significant ways. FTC is primarily a law enforcement agency. It does not engage in pre-market approval of claims, nor does it make regulatory distinctions between product categories (e.g., drugs versus supplements versus foods) or between types of claims (e.g., disease risk claim versus structure-function claim versus drug claim), although those distinctions probably impact which claims FTC chooses to investigate or challenge. FTC authority stems from two provisions of the FTC Act[13]: sections 5 and 12. Section 5 prohibits deceptive and unfair acts or practices in commerce, including deceptive advertising. Section 12 prohibits misleading advertising of foods and other products. Together, these two provisions are the basis for the FTC standard that advertising must be truthful and not misleading and that objective claims must be substantiated before they are made.

FTC Advertising Investigations

When FTC investigates a claim, it asks two basic questions. First, what claim is being conveyed? FTC examines the net impression of an ad and what the consumer takes away, not the intention; it also examines omissions of important information. Rusk remarked that many claims are problematic because they tell only "half the truth." Information that needs to be disclosed must be clear and prominent and not buried in a "fine print footnote." Second, are the claims substantiated? FTC typically requires claims about the efficacy or safety of dietary supplements to be supported with "competent and reliable scientific evidence," defined as "tests, analyses, research, studies or other evidence based on the expertise of professionals in the relevant area, conducted and evaluated in an objective manner by persons qualified to do so, using procedures generally accepted in the profession to yield accurate and reliable results" (FTC, 2001). Rusk explained that while FTC applies a rigorous scientific standard, it is also flexible in its approach. There is no specific number or size of study required, rather the focus is on the quality of the evidence. FTC seeks guidance from experts and generally expects double-blind, placebo-controlled human clinical trials. Rusk noted that the substantiation standard in the FDA draft guidance for dietary supplement claims closely mirrors the FTC substantiation standard (FDA, 2011a).

FTC claims investigations usually involve three steps. First, FTC examines the internal validity of the studies, often with the help of a con-

[13] For more information on the FTC Act, visit the FTC website: http://www.ftc.gov/ogc/stat1.shtm.

sultant. It considers control and blinding; duration (i.e., Does the effect persist?); dose-response relationship (i.e., Is there one?); recognized biological mechanisms (i.e., Is there a biological mechanism that is recognized and understood?); peer review or publication in a reputable journal (not a requirement, but it is a "plus"); and statistical significance and clinical meaning. Second, FTC examines the context of the studies that the company is relying on for substantiation. That is, how do the studies fit into the surrounding literature? If there are any inconsistencies, how are those inconsistencies reconciled? Some claims may need to be qualified in such a way that consumers understand the claim is based on emerging science. Claims should not be made if the weight of the evidence contradicts the claim. Rusk noted that this last point may appear self-evident, but FTC does sometimes encounter situations where a marketer makes a claim based on a single preliminary study. Third, FTC examines the relevance of the science to the claim being made. This is where many companies "slip up," Rusk noted. For example, many weight-loss product claims fail by claiming a much greater weekly weight loss than studies actually show. FTC considers both the amount and the form of an ingredient (e.g., effective dose, strain specificity), the population studied, the degree and nature of the effect, and the strength of the science.

Examples of Recent Actions

Rusk highlighted two recent actions taken by FTC, both consent agreements (i.e., the companies did not admit to any law violations). The first was against advertisements for two Dannon yogurt products, DanActive and Activia. FTC challenged claims that DanActive, with its *L. casei immunitatas* probiotic, helps to prevent colds and flu. It also challenged the claims made during the Jamie Lee Curtis campaign that Activia yogurt, with its *Bifidus regularis* probiotic, relieves irregularity. One of the issues with the Activia campaign was that 8 of 10 studies showed no significant effect at the advertised dose. Rusk noted that FTC was one of many entities investigating the Activia ads and that Dannon settled with both FTC and 39 state attorneys general for a total of $21 million. Increasingly, FTC orders are not purely "cease and desist" orders but also seek monetary relief for the disgorgement of profits from deceptive claims. Where possible, the money is refunded to customers. Otherwise it goes to the U.S. Treasury.

The second action was in response to the Nestlé product Kid Essentials Boost, where the probiotic was embedded in the lining of the straw that comes with the drink. The commission challenged claims that the product prevents upper respiratory infections, helps protect against cold and flu, reduces absences from school, and reduces duration of acute diarrhea in children up to age 13. Nestlé submitted some good evidence to support

its claims, Rusk said, but FTC concluded that the claims went beyond the evidence.

Rusk mentioned recent concern that FTC is raising its substantiation standard. She assured the workshop audience that this is not the case. Rather, the commission is trying to be more specific when it enters into an order with a company it has investigated. In some cases, FTC requires that future claims about specific products go through the FDA approval process. In other cases, it says that claims should be supported by two additional studies conducted by independent researchers. However, these requirements do not reflect a changing standard. Rather, the intention is to be concrete and transparent so that a company under order knows exactly what to expect in terms of compliance.

REGULATORY FRAMEWORKS: THE INDUSTRY EXPERIENCE[14]

Looking through the lens of DuPont, Stuart Craig explored how the changing regulatory landscape is affecting the food industry. DuPont has been a key player in the lactic acid bacteria industry for more than 100 years and works with regulatory agencies worldwide on new legislation and emerging guidelines and requirements related to probiotic safety and efficacy. Indeed, collaborating with regulators to find better ways to ensure food safety and security is a key component of DuPont's focus on food.

As an example of the "paradigm we are in," Craig mentioned the Cheerios labeling controversy. Even though data showed cholesterol reduction, the claim "cholesterol reduction" is a health claim that falls outside the purview of food. In addition to FDA warning letters stating that studies with disease end points support drug claims, not nutrition claims, FDA has made other recent moves that represent significant change for the food industry. Recent FDA actions include the agency's new strategic plan for regulatory science (FDA, 2011a), the Food Safety Modernization Act of 2011,[15] and draft guidance on NDI notifications (FDA, 2011b). FDA's new strategic plan for regulatory science reflects the agency's overall changing priorities: an emphasis on toxicology, personalized medicine, manufacturing and quality, emerging technologies, information sciences, prevention-focused food safety, medical countermeasures, and social and behavioral science. The FSMA legislation is focused on improving the capacity to prevent food safety problems and to detect and respond to food safety problems and on improving the safety of imported food. For a large company such as

[14] This section summarizes the presentation of Stuart Craig.

[15] FDA Food Safety Modernization Act of 2011, Public Law 111-353, 111th Cong. (Jan. 4, 2011).

DuPont, these new challenge are "really not an issue," Craig said, "but for the industry in general, this is going to be quite a change." With respect to the draft guidance on NDI notifications, Craig expects there to be considerable dialogue between the food industry and FDA in the coming years.

The global trend in health claims around food is toward higher safety and efficacy standards. As Craig put it, there is a "higher bar." At the same time, there is also uncertainty around how to scientifically substantiate claims. Craig emphasized the significance of having clear rules and guidance. Gray areas of regulation that are open to interpretation are challenging for everyone involved. Typical questions of regulatory affairs staff include the following: What claims can I make based on this study? Can you make a list of suggested claims we can send to customers? Is this one study enough? If not, why not? If I do another study, will that be enough? Can I study the reduction of XXX and claim the maintenance of YYY or ZZZ? Is this GRAS? Should we notify? Do I need to file an NDI notification?

The EFSA health claim evaluations have drawn on many of DuPont's resources over the past couple of years. Craig noted the high level of EFSA rejections industry-wide, a lack of dialogue, and misunderstanding about what is required of health claims for food products. The challenge has been especially difficult for probiotic and prebiotic claims. While some products have seen success, the overall effect on industry investment has been "chilling." Moreover, the effect is spilling over into other countries.

The "higher bar" for safety and efficacy is difficult for the food industry for two main reasons. First is return on investment. Human clinical studies are expensive, sometimes costing more than $1 million. The food industry model does not compare to the pharmaceutical industry model; with shorter time lines and lower profit margins, protection of research investment in the food industry is lacking. Second, it is more difficult to demonstrate health maintenance than disease intervention. Using the "cholesterol reduction" claim as an example, Craig wondered how a study on cholesterol maintenance would even be designed. Normal subjects should not have elevated cholesterol; they should have normal cholesterol levels, he observed. How does one design a study aimed at evaluating whether a product helps to maintain those normal levels over time? Health maintenance studies need to be longer and involve larger numbers.

Inside DuPont Regulatory and Scientific Affairs

When conducting its own internal scientific substantiation of claims, DuPont tries to use existing tools, such as the Health Canada Claims Substantiation Template, which includes a list of eight items that are scored; the

final score is used to characterize the quality of the study (the eight items are inclusion or exclusion criteria, group allocation, blinding, attrition, exposure or intervention, health effect, statistical analysis, and potential confounders).

DuPont also has its own internal tools for rating evidence, including one developed by Craig that rates each study as A (excellent), B (good), C (weak), or F (does not support) and then rates the overall body of evidence based on the cumulative study ratings. For example, a high-quality study that reported a significant effect but only in the elderly and at a high dose might be rated B because it provides only limited support for a claim that a lower dose is effective in the general adult population. Craig noted that opinions sometimes differ on whether a study should be rated an A, B, or C or even on the overall assessment of the evidence, but at least the tool provides a framework for having a discussion.

Craig remarked that while DuPont is conscientious about scientific substantiation of claims and will provide its clients with analyses of their claims, final responsibility for scientific substantiation lies with the food manufacturer. Because there is no global system for scientific substantiation, with every region operating according to its own rules, what is needed in one country may differ from what is needed in another country. Navigating these complex regulatory differences across the globe is challenging, especially as the global regulatory environment moves toward a higher scientific standard for safety and efficacy, but "we have to rise to that challenge," Craig said. He encouraged more discussion among government, industry, and academia.

THE REGULATORY ENVIRONMENT: A SYNTHESIS[16]

Some of the regulatory challenges addressed by previous workshop speakers stem from the fact that "we are struggling to fit" an emerging science into "an outdated paradigm" for conceptualizing the relationship between food and health, Sarah Roller said. The paradigm recognizes that by virtue of essential nutrients it contributes to the body, food is capable of supporting nutritional needs, promoting normal growth and development, and otherwise affecting the structures and functions of the body. However, the paradigm fails to adequately account for contributions food makes to health through components that are not currently classified as essential nutrients, such as probiotics. The outdated scientific paradigm was codified in the "drug" definition that is part of the FD&C Act adopted in 1938. The drug definition in the FD&C Act continues to play a key role in the regulatory and enforcement policies of FDA and functions to limit the opportuni-

[16] This section summarizes the presentation of Sarah Roller.

ties to convey the health benefits of food products, particularly when the benefits are attributable to components that are not currently recognized to be essential nutrients and/or when the component plays a role in preventing, mitigating, or treating disease. Under the FD&C Act legal framework, marketing claims that represent a product to have disease prevention, mitigation, or treatment-related benefits are prohibited unless the product is an FDA-approved drug and is marketed in compliance with the terms of the FDA approval. Roller pointed out, however, that the regulatory framework FDA has developed for conventional pharmaceutical products is not designed to accommodate foods that can play a useful role in disease prevention, mitigation, or treatment. The obstacles associated with the outdated paradigm are not limited to the FD&C Act framework. The old paradigm has been influential in shaping the legal standards that govern the regulation of food marketing claims under the FTC Act, the federal Lanham Act, other federal statutes administered by the U.S. Department of Agriculture's Food Safety and Inspection Service, and food and drug laws and consumer protection statutes that have been adopted by the 50 states.

Roller called attention to the fact that many state laws allow consumers to sue companies for money damages and injunctive relief when consumers have been misled by the marketing claims companies use to promote their products. In the current legal environment, Roller noted that health benefit claims for food and dietary supplement products are commonly targeted in consumer class action lawsuits filed under state laws. Roller explained that consumer class action lawsuits of this kind are commonly filed in the wake of FDA warning letters and FTC consent orders challenging food marketing claims based on the outdated paradigm. As a result, inadequate federal policies can have a cascading effect under state law, which has costly liability implications for and may deter companies from communicating accurate scientific information about the health benefits of food and dietary supplements, including prebiotics and probiotics. Roller observed that these trends also have the potential to deter industry investment in products that she believes could hold "huge promise for public health."

Roller suggested that it will be necessary to confront the limitations of the FD&C Act "drug" definition as it is applied to food and dietary supplement products to provide sufficient flexibility to allow accurate, substantiated information to be conveyed about the health benefits of these products in commercial contexts, consistent with First Amendment requirements and sound science. Back in 1938, Roller said, the old paradigm probably made sense from a scientific standpoint. At that time, there was little scientific evidence to establish that disease prevention, mitigation, and treatment claims could rightly characterize the benefits of food products. Since that time, science has marched on, and the old "drug" definition and the scientific paradigm upon which it is based no longer

support an adequate regulatory framework for food and dietary supplements, according to Roller.

Under FDA regulations implementing the food provisions of the FD&C Act, FDA has recognized that there are different categories of "food": conventional foods and beverages, chewing gum, food ingredients and components, dietary supplements, food for special dietary use (FSDU), and medical food. Roller referred to Levy's discussion of the challenges that the dietary supplement category creates for probiotic-containing food. She emphasized that further development of FDA policies concerning foods for special dietary use and medical foods is needed and could represent an important but yet "untapped opportunity" for probiotics, in her opinion. FSDU is an old category that was used more often in the past, before the Nutritional Labeling and Education Act of 1990. This category includes foods used for disease-related purposes, specifically for "supplying dietary needs which exist by reason of a physical, physiological, pathological or other condition, including … the conditions of diseases, convalescence, pregnancy, lactation, allergic hypersensitivity to food, underweight, and overweight." Roller suggested that probiotics used to equilibrate the microbiome after antibiotic therapy might be considered FSDU. Medical foods are foods that are to be consumed under a physician's supervision. Lofenalac, an infant formula made for infants with phenylketonuria (PKU), is a classic example of a medical food. In Roller's opinion, between these two food categories, FDA has a "huge amount of discretion."

Changing the Legal Framework

To strengthen the legal framework in the ways that are necessary, Roller urged that the outdated and dualistic "food versus drug" conceptual framework be reconsidered, in view of the distinctions between the cause-and-effect relationships that explain the mechanisms by which pharmaceuticals commonly deliver benefits and those that characterize the mechanisms by which food and dietary patterns deliver benefits. She urged that environmental health and ecological models of causation be given careful consideration in the development of regulatory policies governing food marketing claims for food and dietary supplement products.

In the specific context of claims characterizing the benefits of prebiotic and probiotic components of food and dietary supplement products, Roller outlined several types of claims that she believes would hold promise for product marketing purposes. These include (1) content claims that communicate information about how much of a given prebiotic or probiotic is present in a food, (2) dietary contribution claims that communicate information about the contribution that is made to overall dietary intake of certain prebiotics or probiotics from consumption of particular products

under specified conditions (frequency and quantity), and (3) "ecological" balance and support claims that communicate information about how consumption of a given prebiotic or probiotic affects the microbiome.

Ultimately, Roller expressed hope that the health benefits that are being linked to prebiotics and probiotics with respect to supporting and maintaining a healthy microbiome will help inspire the kinds of policy reforms that are needed to allow food and dietary supplement manufacturers to convey accurate and substantiated information about the benefits of prebiotics and probiotics and the products that contain them, consistent with sound science and First Amendment standards. In Roller's opinion, the current legal framework—the existing "food versus drug" construct that is codified in the FD&C Act—is based on outdated science and is too rigid to allow FDA to account for current science or future advances in science. According to Roller, the current legal framework draws an artificial line between the permissible food benefits of "disease risk reduction" or "health promotion" and the benefits of "disease prevention, mitigation, and treatment," which are categorically reserved for FDA-approved "drugs," even when the distinction may not align with scientific facts. In Roller's opinion, the current situation with probiotics is probably a key moment of opportunity that can allow this long-standing problem to be addressed. Failing to do so could have huge opportunity costs for public health, she added.

Roller speculated on whether it might be possible to adopt an ecological approach to regulating health claims for food products intended to have a beneficial impact on the microbiome, in the same way that environmental law has adopted an ecological approach. For example, fisheries law is based not on individual fish or fish populations, but rather on the environmental conditions required for fish to thrive. She asked, "Does it work for us to think about the microbiome that way?"

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7

Possibilities for the Future

Although research on the microbiome is considered an emerging science, with some areas of research still in their infancy, the field is progressing rapidly. Researchers are making significant headway in understanding not just what the microbiome does, but how the microbiome influences human health and disease—especially through its interaction with diet. Evidence suggests that gut microbes and their human host share much of the same metabolic machinery, with bacteria influencing which dietary components and how much energy their human host is able to extract from its diet. What we eat and drink, in turn, influences the microbiome, with significant implications for disease risk. This growing understanding of the role of diet in microbiome-human interactions is driving interest and investment in probiotic and prebiotic food products as a means to help build and maintain health. Indeed, probiotics are one of the fastest-growing sectors in the global functional food market. Yet, despite this early scientific and market progress, the field faces significant scientific and regulatory challenges. During the last session of the workshop, participants debated ways to move the science forward and drive continued industry investment in microbiome-related product development. This chapter summarizes that discussion. The chapter concludes with a summary of a panel discussion on research priorities that took place earlier in the workshop.

MOVING THE SCIENCE FORWARD: STUDYING HEALTH VERSUS DISEASE

Moderator Fergus Clydesdale initiated the open discussion by observing that the science of the microbiome is focused mostly on associations between the microbiome and disease, not health, and that most dietary interventions intended to have an impact on host biology via their influence on the microbiome (e.g., probiotics) are being studied for their potential to prevent disease, not promote health. He predicted that unless action is taken, the science will continue in that direction—not that it should not. Clydesdale expressed how impressed he was with the science described over the course of the 2-day workshop. The question is, How can additional studies be designed to yield the kind of information needed to substantiate allowable health claims on food products?

The challenge to studying health end points, as opposed to disease end points, is the lack of funding for conducting research in healthy populations, according to Clydesdale. He noted that the National Institutes of Health (NIH) funds disease research, not health research, and that the food industry does not have the kind of money that the pharmaceutical industry has to fund the large clinical studies that would be needed to substantiate health claims. "We have to come up with better ways," he said. He suggested that a public-private partnership might be a good place for dialogue and that the NIH Program on Public-Private Partnerships might be a good place to initiate the conversation. Another participant suggested that some of the many European public-private partnerships might serve as models.

The significance of studying the microbiome in healthy populations extends beyond the health (versus disease) claim implications of doing so. Dan Levy commented on the "huge opportunity" being missed to gain a better understanding of the important role that the microbiome plays in nutrition, by focusing so much on disease. He mentioned the important role that microbially produced short-chain fatty acids play in maintaining colon health. Susan Crockett of General Mills pointed to the "amazing things" that the food industry can do to help people meet recommended nutrition guidelines, implying that the same would be true if there were authoritative guidance on maintaining a healthy microbiome. For example, with respect to fiber, cereals have been reformulated "bit by bit over a period of years" to include more fiber-containing whole grains in an effort to help Americans meet the recommended guidelines for fiber intake. George Fahey agreed that Americans are doing better than in the past, but noted that most Americans still fall short of the recommended daily intake for fiber. Fiber-like prebiotics (i.e., oligosaccharides) could help close that gap. He said, "We have a great opportunity here to meet the guidelines, but we are going to have to be strategic about it." Clydesdale urged the inclusion

of nutrition in the Human Microbiome Project 2 (HMP2), if and when HMP2 is funded.

There was also some discussion about the usefulness of better understanding the impact of the microbiome on host energetics. Fahey suggested that there might be lessons to be learned from studying microbial metabolism in cows and pointed to work done in that area by Marvin Bryant and Robert Hungate. Johan van Hylckama Vlieg suggested that simplified systems models might help to better understand the microbial role in energy metabolism in humans. He said that probably a few core metabolic pathways are major determinants for the impact of the microbiome on host metabolism. Building a systems-level model of gut ecosystem that functions with these core pathways could reduce some of the puzzlement caused by the vastness of the microbiome metagenome and help to better understand clinical observations.

CHANGING THE REGULATORY FRAMEWORK FOR FOOD CLAIMS

With respect to the challenge of health (versus disease) food claims, not all workshop participants agreed that the next best step is to shift the science toward collecting data in healthy populations in order to substantiate those health claims. One audience member agreed that all of the disease-related research on the microbiome described over the course of the 2-day workshop is "great science" and that "the science will continue to go forward," but the greater challenge, he said, is that "we are still left with a 1938 regulatory environment" and regulatory authorities are "hamstrung by whatever the law tells [them]. That is all [they] can act on." He referred to Sarah Roller's suggestion that "maybe we should think about how to change that law." (See the Chapter 6 summary of Sarah Roller's presentation for a description of her views on what she perceives as an outdated regulatory framework for food claims.) Especially given that the science is moving in the disease direction and there will probably come a point when "we may be able to treat or mitigate disease by virtue of foods," the speaker urged that steps be taken now to change the regulatory framework in preparation for the future. Otherwise, how will the value of what scientists discover be communicated? Stuart Craig agreed and said, "The current regulatory environment is paralyzing for industry … we need to start now" (see Figure 7-1).

Peter Turnbaugh cautioned that drawing a hard line between disease and health is "a very dangerous way to think about the way we study" the microbiome. In his opinion, most biologists are interested in fundamental mechanisms. While they hope that their research findings will have helpful applications, that is not the underlying goal of science. Clydesdale

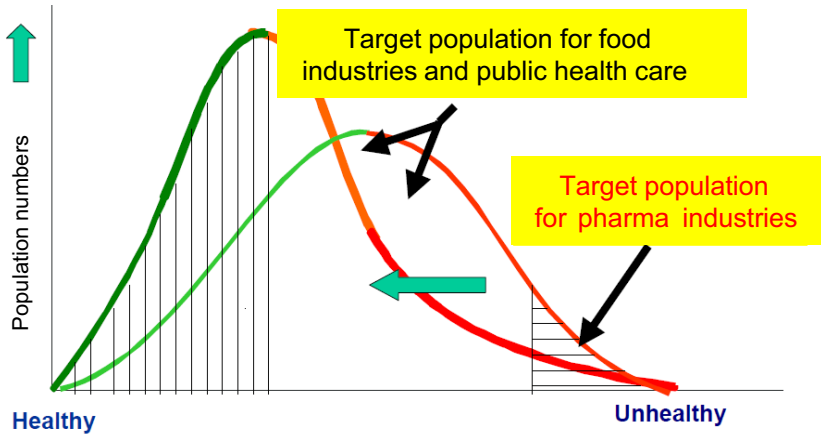

FIGURE 7-1 A key distinction between the food and pharmaceutical industries is that the food industry targets healthy people and people at risk, and the pharmaceutical industry treats unhealthy people. The green arrow represents a desired shift toward health where food can play an important role.
SOURCE: Green and van der Ouderaa, 2003.

responded, "I know all scientists are searching for the truth, and I agree with that goal and that motivation." Yet, the current regulatory environment demands that the "truth" be interpreted in a certain way. Craig added that if a study is conducted on a population of individuals with irritable bowel syndrome, for example, but the food industry cannot communicate that information to consumers, then the information is "stuck." He went on: "If it is great research, but it cannot translate to public health … what value does it truly have?"

Changing the legal regulatory framework for food claims would entail a tremendous challenge. Seppo Salminen commented on the situation in the European Union (EU), where changing regulation would require influencing several hundred EU Members of Parliament. Clydesdale opined that in the United States, he would rather make a recommendation to NIH to "tackle the science" than try to influence Congress. He clarified, "I don't think that there is anyone in the room who would disagree with the fact that we should start talking about it," but he warned not to expect changes in the near future. Craig expressed concern that industry will lose interest if action is not taken. He said that someone needs to start the conversation and "charge forward, instead of just reacting.… It might take 5 or 10 years. Fine. We are in it for the long haul."

So who could start the conversation? An audience member asked whether there were any mechanisms in place to help. For example, what is

the role of the Institute of Medicine's (IOM's) Food Forum? What is the role of the International Life Sciences Institute? What is the role of the Codex Alimentarius Commission? Another audience member referred to Roller's suggestion that lessons might be learned from environmental law. He also mentioned Roller's remarks about potential opportunities in the current law to consider prebiotics or probiotics as food for special dietary use or medical food. Salminen reminded the workshop audience to include the European Union in the dialogue. In a sense, this IOM workshop itself may have served as a starting point. As one audience member said, "I do not recall these conversations really going on seriously at any other meeting."

One audience member suggested that a congressional champion be found in the United States, and perhaps a parliamentary champion in the European Union, to partner with industry and academia as a first step toward renovating the regulatory framework. To attract interest, an argument can be made that being unable to communicate the results of scientific research on food labels is not just a disservice to public health, but also to national competitiveness and global welfare. Another audience member wondered about the role of industry in initiating the conversation. Yet another participant wondered whether the World Health Organization might have a program in place that can serve as a model.

Mary Ellen Sanders agreed that "something needs to be done, something fundamental." However, she cautioned that efforts to change the regulatory framework will take a long time and may not even be successful. Meanwhile, what can be done? Are there ways to approach the regulatory authorities and "nudge" their interpretations of the law around food claims? Roller replied that, yes, there are places in the existing legal framework where the Food and Drug Administration (FDA) has "some running room." However, given all of the other responsibilities that FDA has right now, she predicts that it "is quite doubtful" that it would take this on as well. Historically, solutions to these sorts of problems have come from third parties, where someone else finds the solution and then educates the parties. She concluded, "That is what I would recommend here."

THE MICROBIOME, ENVIRONMENT, AND HEALTH: FUTURE RESEARCH NEEDS

At the end of the first day of the workshop, speakers from the afternoon sessions were asked to identify priorities for future research. This section summarizes their responses. Their responses were not intended to reflect a comprehensive examination of gaps in knowledge, nor do they reflect consensus. The list below reflects individual opinions of workshop speakers.

- *Find shared interest across broad areas of expertise that can drive analysis and interpretation of the massive amounts of sequencing data that are accumulating.*

  - Advances in sequencing technology have generated tremendous amounts of sequencing data, a fact that Karen Nelson, Lita Proctor, and Jennifer Russo Wortman all addressed in different ways. Vincent Young emphasized the importance of finding common ground among broad areas of expertise where experts can work together to drive analysis and interpretation of those data. Too often, he said, sequencing projects end up "half-baked" because researchers are unsure how to proceed.

- *Supplement sequencing studies with mechanistic studies.*

  - Peter Turnbaugh noted that sequencing studies should be complemented with mechanistic studies in animal or perhaps even non-animal model systems. Regardless of the specific research question, without knowing anything about fundamental mechanisms, it is difficult to know what to measure in human studies.

- *Collect prospective data that can inform causality.*

  - Many comments were made throughout the workshop about the abundance of data showing correlations, but not causality, between the microbiome (both composition and activity) and health or disease. Johanna Lampe expressed concern that not enough attention is being directed toward causality. In many cases, it is not clear if a microbial community known to be associated with a disease is a consequence of that disease or a contributing factor. She has been pushing the cancer community to start collecting fecal samples, but the need exists across multiple disease areas, not just cancer.

- *Continue to explore not just the microbiome, but also the metabolome and its role in human health and disease.*

  - Josef Neu urged more studies aimed at understanding the metabolic consequences of the microbiome. For example, researchers have identified microbes associated with necrotizing enterocolitis and other phenomena in preterm babies, but they do not understand the metabolic implications of this association. Johanna

Lampe agreed that researchers, especially nutrition researchers, need to focus more on "who is really active and doing what."

- *Continue to explore the role of commensal microbes in disease.*
  - Both Vincent Young and Richard Darveau touched on the notion that pathogenic disease ensues not just from the presence of a "bad bug," but rather from an imbalance in the indigenous microbial community. Darveau suggested expanding microbiome studies to explore this phenomenon in greater depth.

- *Continue to explore fetal, infant, and pediatric microbiome biology.*
  - Josef Neu urged continued exploration of the relationship between fetal microbial ecology and prematurity. Evidence suggests that contrary to conventional thought, some infants acquire their initial microbiome prior to birth during the third trimester. What microbes are present in the amniotic fluid, and what is their impact on fetal physiology?
  - Neu also urged continued exploration of microbiome differences between cesarean section (C-section) and vaginally delivered infants, given the growing prevalence of C-section deliveries worldwide and the growing number of diseases being associated with C-section delivery (e.g., celiac disease, type 1 diabetes). For example, how does mode of delivery impact development of the immune system in the first year of life?
  - Sharon Donovan urged expanding sample collection in pediatric populations, but in a well-controlled manner. She suggested that research on the infant microbiome could perhaps "piggyback" onto some ongoing or planned large national studies to get a better sense of what is happening in the infant microbiome over time (e.g., the National Children's Study)—for example, by including a fecal sampling protocol. Also, as the Human Microbiome Project moves forward, the inclusion of younger individuals should be considered, Donovan emphasized. Another workshop participant suggested that perhaps some of the longitudinal studies being undertaken around the world, outside the United States, might offer alternative opportunities to collect that type of information. She suggested looking into one of the Scandinavian countries, Japan, or other countries with unified health systems that might make it easier to track study subjects.
  - Donovan also encouraged collection of nutrient intake data as part of any pediatric microbiome study. In her opinion, nutrition

is usually an afterthought. She remarked how "struck" she was by Johanna Lampe's work with phytochemicals. Results from Lampe's work, combined with observations by Peter Turnbaugh that the microbiome can be altered so dramatically in such a short period of time, underscore the important role that nutrition plays.

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A

Workshop Agenda

The Human Microbiome, Diet, and Health
February 22-23, 2012

The Keck Center of the National Academies
500 Fifth Street, NW
Room 100
Washington, DC 20001

Day 1: February 22, 2012—Current and Emerging Knowledge

8:00 am **Registration**

8:30 **Welcome and Introductions**
Gordon Jensen, *Workshop Planning Committee Chair*
Harvey Fineberg, *Institute of Medicine*

8:45 **Keynote: The Future Impact of Beneficial Microbes and Gut Health**
Karen Nelson, *J. Craig Venter Institute*

SESSION 1—THE STUDY OF THE HUMAN MICROBIOME

9:15 **Session 1 Introduction**
Moderator: Cindy Davis, *Office of Dietary Supplements, National Institutes of Health (NIH)*

9:20 **Defining the Human Microbiome**
Lita Proctor, *Human Microbiome Project, National Human Genome Research Institute (NHGRI), NIH*

10:10 **Break**

10:20 **Tools and Models of Assessment for the Microbiome**
Jennifer Russo Wortman, *Broad Institute*

10:50 **Metabolome and Microbiome**
Jeremy Nicholson, *Imperial College London*

11:35 **Panel Discussion with Session 1 Speakers**

12:05 pm **Lunch**

SESSION 2—INTERACTION BETWEEN THE MICROBIOME AND HEALTH AND ENVIRONMENT

1:00 **Session 2 Introduction**
Moderator: Jennifer Brulc, *General Mills, Inc.*

1:05 **Overview of Pediatric Clinical Implications and Interventions**
Josef Neu, *University of Florida*

1:35 **Impact of Microbiome on Oral Health and Disease**
Richard Darveau, *University of Washington*

2:05 **Impact of Microbiome on Gastrointestinal Health**
Vincent Young, *University of Michigan*

2:35 **Diet, Obesity, and the Gut Microbiome**
Peter Turnbaugh, *Harvard University*

3:05 **Break**

SESSION 3—INFLUENCE OF THE MICROBIOME ON DIET AND DIETARY COMPONENTS

3:15 **Session 3 Introduction**
Moderator: Bruce German, *University of California, Davis*

3:35 **Host-Microbe Interactions in the Perinatal Period: Role of Early Nutrition**
Sharon M. Donovan, *University of Illinois*

4:05 **Microbial Metabolites of Dietary Components**
Johanna Lampe, *Fred Hutchinson Cancer Research Center*

SESSION 4—SUMMARY PANEL

4:35 **Review and Discussion with Day 1 Speakers**
Moderator: Gordon Jensen, *Pennsylvania State University*

5:35 **Adjourn**

Day 2: February 23, 2012—Nutrition Translation: From Lab Bench to Food Product

8:00 am **Registration**

SESSION 5—INFLUENCE OF DIET AND DIETARY COMPONENTS ON THE MICROBIOME

8:30 **Session 5 Introduction**
Moderator: David Mills, *University of California, Davis*

8:40 **Human Breast Milk**
Bruce German, *University of California, Davis*

9:05 **The Resistome as a Driver of the Microbiome**
Ellen Silbergeld, *Johns Hopkins University*

9:30 **Probiotic Mechanisms of Action**
James Versalovic, *Baylor College of Medicine*

9:55 **Prebiotic Mechanisms of Action**
George Fahey, *University of Illinois*

10:20 **Translation of Probiotic Science into Probiotic Foods**
Mary Ellen Sanders, *Dairy & Food Culture Technologies*

10:45 **Developing Delivery Systems**
D. Julian McClements, *University of Massachusetts Amherst*

11:10 **How the Microbiome Revolution Fuels Function Food Research—Practical Examples and Prospects**
Johan van Hylckama Vlieg, *Danone Research Center*

11:35 **General Discussion**

12:05 pm **Lunch**

SESSION 6—SOCIETAL AND POLICY IMPLICATIONS

1:05 **Session 6 Introduction**
Moderator: Mary Ellen Sanders, *Dairy & Food Culture Technologies*

1:10 **How Americans Eat and Drink to Improve Health**
Darren Seifer, *NPD Group*

1:35 **Consumer Insights from the Industry Perspective**
Peggy Steele, *DuPont Nutrition and Health*

2:10 **Probiotic and Prebiotic Health Claims in Europe: Scientific Assessment and Requirements**
Seppo Salminen, *University of Turku, Finland*

2:35 **Evaluation of Viable Microbes Using Regulatory Requirements Developed for Non-Viable Ingredients**
Dan Levy, *U.S. Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition*

3:00 **Break**

3:10 **Health Claims and False Advertising**
Michelle Rusk, *Federal Trade Commission*

3:35 **Regulatory Frameworks—The Industry Experience**
Stuart Craig, *DuPont Nutrition and Health*

4:00 **Synthesis of the Regulatory Environment**
Sarah Roller, *Kelley Drye & Warren LLP*

SESSION 7—SUMMARY PANEL: RESEARCH AND PRODUCT OR TECHNOLOGY DEVELOPMENT DIRECTIONS FOR THE FUTURE

4:25 **Open Discussion with All Speakers and Audience**
Discussant: Fergus Clydesdale, *University of Massachusetts Amherst*

5:30 **Adjourn**

B

Speaker Biographical Sketches

Jennifer Brulc, Ph.D., is senior scientist at the General Mills Bell Institute of Health and Nutrition. Prior to working at General Mills, Dr. Brulc completed her postdoctoral work at the Institute for Genomics and Systems Biology at Argonne National Laboratory and the University of Chicago, where she studied the use of bioinformatics and newly developed genomics technologies both to observe the microbial impact on human gastrointestinal disease states and to assess metabolic potential differentiation and microbial influence on ecosystem development in topsoil environments. This research built upon her Ph.D. research under Dr. Bryan A. White, in which Dr. Brulc focused on the divergence of complex microbial communities and their resulting community interactions on host nutrition and diet adaptation in mammalian gastrointestinal tracts as related to efficient fiber degradation, using second-generation DNA sequencing technologies. Dr. Brulc received her Ph.D. from the University of Illinois at Urbana-Champaign.

Fergus M. Clydesdale, Ph.D., is distinguished university professor, Department of Food Science, University of Massachusetts Amherst, and director of the University of Massachusetts Food Science Policy Alliance. From 1988 to 2008, he was head of the Department of Food Science, which at the time of his retirement was ranked the top department in the university in student satisfaction and recently ranked the top department in the country by the National Research Council. He is a fellow of five premier societies in the field of food science and nutrition and editor of *Critical Reviews in Food Science and Nutrition*, and he has published some 375 scientific articles and coauthored or edited 20 books. Dr. Clydesdale also has served

on or chaired numerous committees of the Institute of Food Technologists (IFT), Food and Drug Administration (FDA), International Life Sciences Institute (ILSI), International Food Information Council (IFIC), and the National Academies as well as serving on the Food and Nutrition Board and the 2005 Dietary Guidelines Advisory Committee. He is the recipient of numerous awards, including IFT's highest honor, the Nicolas Appert Award.

Stuart A. S. Craig, Ph.D., is director, regulatory and scientific affairs, for DuPont Nutrition and Health. His current responsibilities include the safety, physiological benefits, and regulatory status of food ingredients. His focus is on carbohydrates (dietary fiber, prebiotics, glycemic response) and methyl metabolism in sports nutrition and the prevention of chronic disease. Dr. Craig has previously held research positions at Kansas State University, Nabisco, Pfizer, and Cultor. During that time he has conducted studies and led groups in the areas of biochemistry, physical chemistry, food safety, microbiology, sensory science, and nutrition. He has authored or co-authored numerous papers in peer-reviewed journals and book chapters. Dr. Craig is an inventor on several patents and has presented extensively in the United States, Europe, and Asia. He is past president of AACC International; past chair of the Starch Roundtable; and a member of the American Chemical Society, Royal Society of Chemistry, Institute of Food Technologists, and American Society for Nutrition. Dr. Craig is active with the ILSI, where he is past chair of the Food, Nutrition, and Safety Program. He received his B.Sc. and Ph.D. in biochemistry from Heriot-Watt University, Edinburgh, Scotland.

Richard Darveau, Ph.D., is professor and chair in the Department of Periodontics at the University of Washington Dental School in Seattle. He is funded by the National Institute of Dental and Craniofacial Research and is actively exploring the relationship between the oral flora and periodontal health and disease in his laboratory. Dr. Darveau has more than 100 publications, holds several patents, and has served on numerous National Institutes of Health (NIH) study sections as well as scientific advisory boards for dental health–related companies. Dr. Darveau was awarded the International Association of Dental Researchers 2007 Basic Research in Periodontal Disease Award. He has been an invited speaker for numerous national and international meetings. He received his Ph.D. in microbiology from Washington State University, did a postdoctoral fellowship with the Canadian Cystic Fibrosis Foundation for 2 years, and then worked in industry with Abbott Laboratories and Bristol-Myers Squibb for 14 years as a research scientist and group leader.

Cindy D. Davis, Ph.D., is the director of grants and extramural activities in the Office of Dietary Supplements (ODS) at NIH. In this position she actively engages and encourages partnerships with other NIH institutes and centers to facilitate funding of grants that are of high relevance to ODS mission and goals. Before coming to ODS in November 2011, Dr. Davis was a program director in the Nutritional Sciences Research Group at the National Cancer Institute (NCI), where she had worked since 2002. Prior to NCI, she was a research nutritionist at the U.S. Department of Agriculture (USDA) Grand Forks Human Nutrition Research Center, where her research focused on the effect of trace minerals on cancer susceptibility. Dr. Davis has published more than 100 peer-reviewed journal articles and 11 invited book chapters. She is on the editorial board of the *Journal of Nutrition and Food Sciences*, *Journal of Nutrition and Metabolism*, and *Nutrition Reviews*. Dr. Davis received her Ph.D. in nutrition with a minor in human cancer biology from the University of Wisconsin. She completed her postdoctoral training at the Laboratory of Experimental Carcinogenesis at NCI.

Sharon Donovan, Ph.D., R.D., is professor and Melissa M. Noel Endowed Chair in Nutrition and Health at the University of Illinois. She was the first recipient of the Melissa M. Noel Endowed Chair at the University of Illinois. She served as director of the Division of Nutritional Sciences Interdisciplinary Graduate Program from 1999 to 2009. Her research focuses on pediatric nutrition, with an emphasis on optimization of neonatal intestinal development. She compares the biological effects of human milk and infant formulas on intestinal function in human infants, in neonatal piglets, and in various models of intestinal disease. Dr. Donovan has published more than 100 peer-reviewed publications, review articles, and conference proceedings. She is the recipient of several awards in recognition of her research, including the Mead Johnson Award and the Norman A. Kretchmer Award from the American Society for Nutrition. Dr. Donovan received her B.S. and Ph.D. in nutrition from the University of California, Davis, and completed her postdoctoral fellowship in pediatric endocrinology at Stanford University School of Medicine.

George C. Fahey, Jr., Ph.D., is professor emeritus of animal sciences and Kraft Foods endowed professor emeritus of nutritional sciences at the University of Illinois at Urbana-Champaign, where he has been a faculty member since 1976. His area of research is carbohydrate nutrition, including work on dietary fibers, oligosaccharides, resistant starch, and novel polysaccharides. An overarching theme of his program is gastrointestinal tract health and the role of carbohydrates in the improvement of indexes of gut health. Glycemic control and its relationship to diabetes constitute

another major area of study. Dr. Fahey has advised more than 90 individuals to the successful completion of their graduate degree programs or postdoctoral research associate positions. He serves on a number of editorial boards and on several scientific advisory boards for companies and professional organizations. He is a frequent speaker at both academic and industry events and has published extensively in his research areas. He has won research awards from his department, college, and university, as well as national and international awards.

Bruce German, Ph.D., is professor in the Department of Food Science and Technology at the University of California, Davis, and director of the Foods for Health Institute at the university. He joined the faculty at UC Davis in 1988; in 1997, he was named the first John E. Kinsella Endowed Chair in Food Nutrition and Health. His research interests include the structure and function of dietary lipids, the role of milk components in food and health, and the application of metabolic assessment to personalized health. He received his Ph.D. from Cornell University.

Johanna W. Lampe, Ph.D., R.D., is a member and associate division director in the Public Health Sciences Division at the Fred Hutchinson Cancer Research Center and a research professor in the Department of Epidemiology at the University of Washington in Seattle. Her research program addresses the effect of plant-food constituents on cancer susceptibility in humans and the interindividual variation in gut bacterial metabolism of phytochemicals. Her group uses controlled dietary interventions to evaluate cancer biomarker response to diet and specific phytochemicals and diet-induced changes in the gut microbiome. Dr. Lampe received her Ph.D. in nutritional sciences, with a minor in biochemistry, from the University of Minnesota and trained as a postdoctoral fellow in epidemiology at the University of Minnesota before joining the faculty at Fred Hutchinson Cancer Research Center in 1994.

Dan D. Levy, Ph.D., is a microbiologist and supervisor of the New Dietary Ingredient Review Team in the Division of Dietary Supplement Programs at the FDA Center for Food Safety and Applied Nutrition (CFSAN). Prior to evaluating the safety of dietary supplement ingredients in pre-market new dietary ingredient notifications, he studied the molecular genetics of foodborne pathogens as a research scientist at CFSAN. He has authored more than 20 peer-reviewed scientific publications; his current research collaborations include development of genetic methods for the identification of live microbial "probiotic" food ingredients, validation of the comet assay, and interpretation of data from genetic toxicology testing methods. He co-chaired the 2010 New York Academy of Sciences Conference "Probiotic

Foods and Supplements" and was FDA project officer for the Agency for Healthcare Research and Quality (AHRQ) evidence report on the safety of probiotics. Dr. Levy received his Ph.D. from the New York University Sackler Institute of Biomedical Sciences and did his postdoctoral training at NCI and the Center for Nuclear Studies in Grenoble, France.

David Julian McClements, Ph.D., is a professor in the Department of Food Science at the University of Massachusetts Amherst. He specializes in the areas of food biopolymers and colloids and, in particular, the development of food-based structured delivery systems for active components. Dr. McClements has received awards from the American Chemical Society, American Oil Chemists Society, Institute of Food Technologists, and University of Massachusetts in recognition of his scientific achievements. His research has been funded by grants from the U.S. Department of Agriculture, National Science Foundation, U.S. Department of Commerce, Dairy Management Incorporated, and the food industry. He is a member of the editorial board of a number of journals and has organized workshops and conferences in the field of food colloids, food emulsions, and delivery systems. Dr. McClements received his Ph.D. in food science at the University of Leeds. He then did postdoctoral research at the University of Leeds, University of California, Davis, and University College Cork in Ireland.

David Mills, Ph.D., is professor in the Department of Viticulture and Enology in the Robert Mondavi Institute for Wine and Food Sciences at the University of California, Davis. Dr. Mills studies the molecular biology of lactic acid bacteria in food and beverage fermentations or as probiotics in intestinal health. He has served as a Waksman Foundation Lecturer for the American Society for Microbiology (ASM) and currently serves as an associate editor for the journal *Microbiology*. He has held various positions, including chair, with the food microbiology division of ASM. In 2010, Dr. Mills was awarded the Cargill Flavor Systems Specialties Award from the American Dairy Science Association.

Karen Nelson, Ph.D., is president of the J. Craig Venter Institute (JCVI), where she has worked for the past 16 years. Prior to being appointed president, she held a number of other positions at the institute, including director of JCVI's Rockville, Maryland, campus, and director of human microbiology and metagenomics in the Department of Human Genomic Medicine at JCVI. Dr. Nelson has extensive experience in microbial ecology, microbial genomics, microbial physiology, and metagenomics. Since joining the JCVI legacy institutes, she has led several genomic and metagenomic efforts and the first human metagenomics study on fecal material derived from three individuals that was published in 2006. Additional ongoing studies in her

group include metagenomic approaches to study the ecology of the gastrointestinal tract of humans and animals, studies on the relationship between the microbiome and various human and animal disease conditions, reference genome sequencing and analysis primarily for the human body, and other -omics studies. Dr. Nelson received her undergraduate degree from the University of the West Indies and her Ph.D. from Cornell University.

Josef Neu, M.D., is professor of pediatrics and director of the Neonatology Fellowship Training Program in the Division of Neonatology at the University of Florida. He has served on the Council for the Organization of Neonatal Training Program Directors (ONTPD) for the past 3 years and has recently completed 2 years as national chairman of ONTPD. Dr. Neu is internationally recognized for his research in developmental gastroenterology and nutrition and has most recently focused his research efforts on the microbiome. He is currently funded by NIH to study the developing microbiome and to discover biomarkers in babies at risk for developing necrotizing enterocolitis. This involves a multicenter evaluation of intestinal microbiota using novel non–culture-based technologies. He did his medical school training at the University of Wisconsin, was a pediatric resident at Johns Hopkins University, and was a postdoctoral neonatology fellow at Stanford University.

Jeremy Nicholson, Ph.D., is head of the Department of Surgery and Cancer at the Imperial College London. He leads the research program of the Imperial College Healthcare Trust (National Health Service) Surgery and Cancer Clinical Programme and the Imperial Area Health Authority Biomedical Research Centre Programme in Stratified Medicine for optimizing translational medicine for patient safety and health care delivery. Dr. Nicholson has authored more than 500 peer-reviewed papers and many other articles or patents on molecular aspects of complex system failure and the role of the microbiome-host metabolic signaling in disease etiopathogenesis. He is a fellow of the Royal College of Pathologists, the Royal Society of Chemistry, the Institute of Biology, and the UK Academy of Medical Sciences. He is on the editorial board of several major international science journals and is consulting editor for the *Journal of Proteome Research*. He is a consultant to many pharmaceutical and health care companies in the United Kingdom, continental Europe, and the United States and is a founding director of Metabometrix, an Imperial College spin-off company specializing in molecular phenotyping, clinical diagnostics, and toxicological screening. He received his Ph.D. from London University, working on the application of analytic electron microscopy and the applications of energy dispersive X-ray microanalysis in molecular toxicology.

Lita Marie Proctor, Ph.D., is the coordinator of the NIH Common Fund Human Microbiome Project as well as program director in the National Human Genome Research Institute. Prior to her current position, Dr. Proctor held appointments at Florida State University and at the University of California, Santa Cruz. She also served as a program director at the National Science Foundation (NSF). Dr. Proctor obtained her Ph.D. in oceanography from the State University of New York at Stony Brook. She trained as an NSF postdoctoral fellow in molecular genetics at the University of California, Los Angeles.

Sarah Roller, J.D., R.D., M.P.H., is a partner with the Washington, DC, law firm Kelley Drye & Warren LLP and chair of the Food and Drug Law Practice. She focuses her practice on the representation of U.S. and global companies and industry trade organizations engaged in the development, manufacture, import, export, distribution, and marketing of food and beverage products and components and the associated regulatory issues involved at the international, federal, and state levels. Ms. Roller assists companies in developing legal risk management strategies and compliance programs, establishing that product formulations and ingredients meet regulatory agency standards, and ensuring that product benefit claims are adequately substantiated by scientific evidence. She previously worked as a life sciences policy analyst with the Congressional Research Service and served as a clinical research nutritionist with the Mt. Sinai Hypertension Trial conducted by Mt. Sinai Hospital and the University of Minnesota. Ms. Roller is a member of the American Bar Association; American Society for Law, Medicine, and Ethics; American Public Health Association; Institute of Food Technologists; and American Dietetic Association. She is a registered dietitian and received her J.D. from the George Washington University Law School.

Michelle Rusk, J.D., is a senior staff attorney in the Division of Advertising Practices, Bureau of Consumer Protection, at the Federal Trade Commission (FTC). The division is responsible for regulating national advertising matters, including claims about foods, over-the-counter (OTC) drugs, dietary supplements, cosmetics, alcohol, tobacco, and environmental products. Ms. Rusk has been responsible for coordination of FTC enforcement activities for food and dietary supplement advertising and has been involved in various policy matters related to food marketing during her 20-year career at the commission. Following passage of the Nutrition Labeling and Education Act of 1990, she worked on developing an FTC policy on food advertising to harmonize with FDA and USDA regulations. She was involved in writing FTC's 1994 *Enforcement Policy Statement on Food Advertising*. Ms. Rusk also developed FTC's 1998 *Dietary Supplements:*

An Advertising Guide for Industry. She was the 1999 recipient of the commission's Paul Rand Dixon Award for her work in the dietary supplement area. She is currently working on issues related to childhood obesity and is part of an interagency working group charged by Congress with developing recommendations for nutritional standards for foods marketed to children. Ms. Rusk joined FTC from private practice in 1990. She graduated from Harvard University and received her J.D. from the Georgetown University Law Center.

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Mary Ellen Sanders, Ph.D., is a consultant in the area of probiotic microbiology. Her recent focus has been on efficacy substantiation, microbiology, and regulatory issues pertaining to probiotics. She has coordinated or collaborated on clinical studies to validate probiotic efficacy, served on GRAS (generally recognized as safe) determination panels, participated in a working group convened by FAO-WHO (Food and Agriculture Organization-World Health Organization) to make recommendations to Codex for guidelines for use of probiotics, and served on the World Gastroenterology Organisation Guidelines Committee preparing guidelines for the use of probiotics and prebiotics for gastroenterologists. Dr. Sanders serves as executive director of the International Scientific Association for Probiotics and Prebiotics (ISAPP; www.isapp.net). She also hosts a website, along with the California Dairy Research Foundation, that provides objective, evidence-based information on probiotics for consumers and professionals (www.usprobiotics.org).

Darren Seifer is the food and beverage industry analyst for the NPD Group, a leading market research company. He provides insights based on NPD's food-related research to organizations and companies across the country. Prior to joining NPD in 2007, he was an analyst with Information

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Ellen Silbergeld, Ph.D., is professor in epidemiology, environmental health sciences, and health policy and management at Johns Hopkins University. Her research and professional activities bridge science and public policy, with a focus on the incorporation of mechanistic toxicology into environmental and occupational health policy. Her areas of current focus include cardiovascular risks of arsenic, lead, and cadmium; immunotoxicity of mercury compounds; and the health and environmental impacts of industrial food animal production. She has served as a science adviser for several federal agencies, including the Environmental Protection Agency and the Centers for Disease Control and Prevention, as well as international organizations such as the World Bank and the United Nations Environment Programme. She is editor-in-chief of *Environmental Research* and serves on the editorial board of several high-impact journals. She has received numerous awards, including a lifetime achievement award from the Society of Toxicology, the Barsky Award of the American Public Health Association, and a "Genius Award" from the MacArthur Foundation. Dr. Silbergeld is trained in environmental engineering and toxicology and holds a Ph.D. in environmental engineering from Johns Hopkins University.

Peggy Steele, M.S., is a global business director within the Nutrition and Health Division of DuPont. Ms. Steele has more than 20 years of experience in the food and dairy industry, where she has held positions in business development, product management, research and development, and quality assurance. She has her B.S. degree in nutrition from San Jose State University and her M.S. degree in food science from the University of Minnesota.

Peter J. Turnbaugh, Ph.D., is a Bauer fellow in the FAS Center for Systems Biology at Harvard University. Since 2004, his research has focused on the trillions of microbes that colonize our adult bodies. This human "microbiome" encodes metabolic capacities that remain largely unexplored but include the degradation of otherwise indigestible components of our diet. Dr. Turnbaugh and his research group combine metagenomics, anaerobic microbiology, and gnotobiotic (germ-free and colonized) mouse systems to study the diversity and function of the human gut microbiome. This work

has focused primarily on the interactions among host diet, energy balance, and the gut microbiome, leading to a new model for the role that microbes can play in nutrition and obesity. Currently, his team is focusing on the metabolism of orally administered therapeutic drugs by the distal gut microbiome. He received a B.A. in biochemistry, biophysics, and molecular biology from Whitman College and a Ph.D. in microbial genomics from Washington University in St. Louis.

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James Versalovic, M.D., Ph.D., is head of the Department of Pathology, chief of the Pathology Service, and director of the Texas Children's Microbiome Center. He serves as the Milton J. Finegold Professor of Pathology and Immunology and also is professor of pediatrics, molecular and human genetics, and molecular virology and microbiology at Baylor College of Medicine (BCM). He is co-director of the medical scientist (M.D.-Ph.D.) training program at BCM. He pursued clinical pathology and microbiology residency training at Massachusetts General Hospital and Harvard Medical School. Dr. Versalovic is board certified in clinical pathology and molecular genetic pathology. He is editor-in-chief of the *Manual of Clinical Microbiology* and editor of *Therapeutic Microbiology: Probiotics and Related Strategies*. As a principal investigator, his primary research interests include the human microbiome, probiotics, medical and molecular microbiology, innate immunity, digestive diseases, and gastrointestinal physiology. His research program is supported by NIH (R01 and Roadmap funding). Dr. Versalovic has authored 88 primary manuscripts, 30 book chapters, and 2 patents. He received the Lansky Award as a national leader in pathology under the age of 45 from the College of American Pathologists Foundation. He has also received the BioGaia Ivan Casas Probiotics Research Award and the BCM Graduate School of Biomedical Sciences Distinguished Alumnus Award. He received his M.D. with honors and his Ph.D. in cellular and molecular biology at BCM.

Jennifer Russo Wortman, M.S., is director of microbial informatics and is responsible for oversight of the bioinformatics, genome analysis, and software

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Vincent B. Young, Ph.D., M.D., is an associate professor in the Department of Internal Medicine, Infectious Diseases Division, and the Department of Microbiology and Immunology at the University of Michigan Medical School. His research is directed at understanding the role of bacteria that inhabit the gastrointestinal tract and how they influence the health status of the host. Researchers in Dr. Young's lab study the role of what would traditionally be considered "pathogenic bacteria" in gastrointestinal (GI) illness. In addition, they also examine how the population structure of indigenous GI microbiota can influence host-pathogen interaction and how changes in the community structure of indigenous microbiota can lead to pathogenic states. This research is being conducted both with material from human subjects and with animal models of disease. Dr. Young received his B.S. from the Massachusetts Institute of Technology and his M.D. and Ph.D. from Stanford University. He completed his clinical training in internal medicine and infectious diseases at Massachusetts General Hospital.

C

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D

Abbreviations and Acronyms

AHRQ	Agency for Healthcare Research and Quality
BCFA	branched-chain fatty acid
BMI	body mass index
CFU	colony-forming unit
COPD	chronic obstructive pulmonary disease
DIO	diet-induced obesity
DNA	deoxyribonucleic acid
DRI	dietary reference intake
DSHEA	Dietary Supplement Health and Education Act
EFSA	European Food Safety Authority
EPAS1	endothelial PAS domain-containing protein 1
EU	European Union
FAO	Food and Agriculture Organization
FD&C Act	Federal Food, Drug, and Cosmetic Act
FDA	U.S. Food and Drug Administration
2'-FL	2'-fucosyllactose
FMP	fermented milk product
FSDU	food for special dietary use
FSMA	Food Safety Modernization Act
FTC	Federal Trade Commission

GABA	gamma-aminobutyric acid
GalOS	galactooligosaccharide
GDP	gross domestic product
GI	gastrointestinal
GMO	genetically modified organism
GRAS	generally recognized as safe
GWAS	genome-wide association studies
HMO	human milk oligosaccharide
HMP	Human Microbiome Project
HUMAnN	HMP Unified Metabolic Analysis Network
IBD	imflammatory bowel disease
IBS	irritable bowel syndrome
ICU	intensive care unit
IFIC	International Food Information Council
IgA	immunoglobulin A
IHMC	International Human Microbiome Consortium
IL	interleukin
IND	Investigational New Drug
IOM	Institute of Medicine
IRB	institutional review board
ITC	isothiocynate
JCVI	J. Craig Venter Institute
LDL	low-density lipoprotein
LPS	lipopolysaccharide
LRH	liver receptor homologue
MAP	mitogen-activated protein
MDA	multiple displacement amplification
mRNA	messenger ribonucleic acid
MWAS	metabolome-wide association studies
NDI	new dietary ingredient
NDO	nondigestible oligosaccharide
NEC	necrotizing enterocolitis
NIH	National Institutes of Health
NMR	nuclear magnetic resonance
NSF	National Science Foundation
OTU	operational taxonomic unit

PCR	polymerase chain reaction
PDX	polydextrose
PKU	phenylketonuria
qRT	quantitative real-time
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
RYGB	Roux-en-Y gastric bypass
SCF	soluble corn fiber
SCFA	short-chain fatty acid
TF	transcription factor
TJP1	tight junction protein 1
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF	tumor necrosis factor
USDA	U.S. Department of Agriculture
WHO	World Health Organization